PLENARY

Attack and Defend: The Function and Evolution of Bioactive or Toxic Metabolites

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"…there is a constant struggle between the instinct of one to escape its enemy and of the other to secure its prey…."

As competition between species intensifies, an organism will adopt any advantage it can acquire to survive, and often this means some form of chemical warfare. This was recognized very early by Darwin who observed that there is a constant struggle to survive—even dominate (Darwin, see Romanes, 1884).

Today, this is a well-recognized concept, and is illustrated by many examples in nature. For example, newts of the genus *Taricha* survive in a hostile world by lacing their skin with tetrodotoxin, a neurotoxin found in other amphibians as well as fish. Normally resistant to predator snakes, the newts are prey to the garter snake *Thamnophis sirtalis* that has immunity to the toxin (Geffeney *et al.,* 2002). This immunity is presumed to arise by alteration of the target Na channels in the snake, but with this immunity comes a penalty. Those snakes resistant to tetrodotoxin are slower-moving than their non-resistant counterparts, making them in turn, more susceptible to predation. From this we learn some simple facts: First, the definition of toxin is variable, depending on the point of view. Obviously, the potent neurotoxin is regarded by the newt as a benign and beneficial compound. From the point of view of a snake *sensitive* to tetrodotoxin, it is clearly a toxin. Second, we learn that acquisition of resistance is likely to carry a metabolic or physiological penalty—in this case a slower moving snake.

Another fact emerges: The strategy of the tetrodotoxin-laced newt to avoid predation, although effective much of the time, is not totally infallible. What other options are there for an organism to survive? It turns out there are several: For many species, predator avoidance could simply require escape—either by running, swimming, or flying. For higher mammals, the immune defense system is the primary means of defense, but organisms lacking such a sophisticated approach often resort to chemical defense (Mebs, 2001). Indeed, every organism lacking an immune system produces compounds known as secondary metabolites, which may be involved in some aspect of chemical defense (Hadacek, 2002). Well over 150,000 secondary metabolites have been identified from land-based or marine microbes, plants, and invertebrates, and the list grows daily.

Unlike primary metabolites, secondary metabolites are compounds that do not appear to play an essential role in the survival of a cell— at least in terms of its regular function. A myriad of studies aimed at identifying the role of secondary metabolites in a cell have uncovered many possible functions. An offensive function is defined as one that will help the organism to capture prey. A defensive function is designed to help the organism avoid a predator, and here the activity can be either constitutive or inducible (Harborne, 1999). In the former case, the defensive compound(s) is made all the time, whether a predator is nearby or not. In the second case, a defensive compound is produced in response to an initial attack. The latter approach is more economical in the sense that compounds are only produced when needed, whereas in the other case, the compounds are produced whether there is an immediate requirement for them or not. In addition to an offensive or defensive role, other functions assigned to secondary metabolites include the following:

- Offense (capture of prey)
- Defense (constitutive or inducible)
- Reproduction (pheromones)
- Warning or signaling compounds
- Siderophores
- Photoprotectants (constitutive or inducible)
- Osmoregulators

The identification of so many different secondary metabolites displaying such a range of functions poses the question of how secondary metabolism evolved. The remarkable variety and numbers of metabolites can be answered in a trivial sense by a quote from the Greek philosopher Herodotus who noted that

"Given enough time, everything possible will happen."

Indeed, this seems to be the case in the evolution of secondary metabolism. Thus an organism can acquire metabolites by dietary acquisition of metabolites from another organism, and can introduce further structural variety by metabolic modification or alteration of these dietary metabolites. Secondary metabolites may be acquired through a symbiotic relationship with another producing organism such as a bacterium. However, the main source of secondary metabolites in most organisms is through de novo synthesis following the mutation and recombination of primary metabolic pathways, duplication of modified primary pathways, as well as by lateral or horizontal transfer of these biosynthetic genes from one organism to another (Pichersky and Gang, 2000). Following such a gene transfer, even more structural variety can be obtained within the new host by invoking an additional series of structural modifications to the basic molecular product.

Thus most secondary metabolic pathways are believed to have arisen from mutations of primary pathways. A case in point is the origin of polyketide synthesis, which ac-

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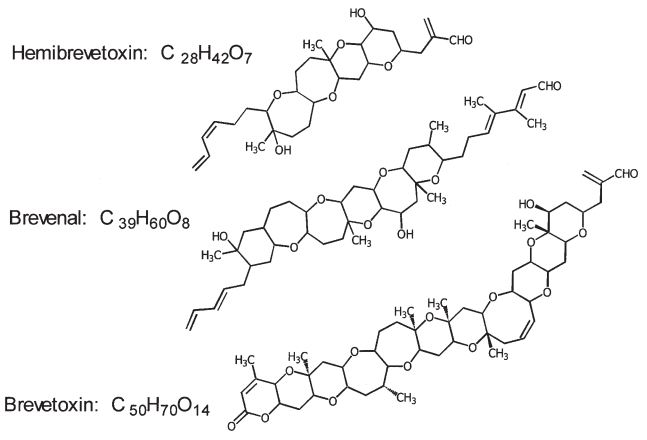


Figure 1 An example of a putative extension biosynthetic pathway. Each polyether metabolite is produced by the same dinoflagellate *Karenia brevis*.

counts for the largest group of secondary metabolites, and is believed to have arisen from the primary process of fatty acid biosynthesis. The basic building blocks for fatty acids are acetate units that are joined together by a complex enzyme system called a fatty acid synthase (FAS). Mutations of these enzymes yields the polyketide synthase (PKS) enzymes, responsible for the production of partly reduced products known as polyketides, and further modification of these polyketides by so-called "tailoring enzymes" leads to even more structural complexity (Staunton and Weissman 2001). Similarly, mutations of the isoprenoid synthase enzymes involved in sterol and carotenoid biosynthesis lead to a wide range of secondary metabolites known as the isoprenoids, and mutation or modification of the shikimate biosynthetic pathway to aromatic compounds results in the production of new alkaloids. Another large group of secondary metabolites known as non-ribosomal peptides are composed of D- and L- amino acids and non-protein amino acids that are assembled by a protein complex known as a non-ribosomal synthase (NRPS). The modular arrangement of the NRPS is similar to that of FAS or PKS, and involves adaptations of primary processes such as adenylation, and acyl carrier protein domains (Marahiel *et al.,* 1997).

Given these basic pathways to secondary metabolites, it is possible to envisage that further chemical diversity is generated by additional mutation, domain swapping, and recombination of biosynthetic genes. Three routes to generate chemical diversity have been noted (Stone and Williams, 1992).

- Extension of existing pathways
- Divergence of existing pathways
- Convergence of existing pathways.

An extension of a biosynthetic pathway is illustrated by consideration of three polyether metabolites (Fig.1) isolated from the same dinoflagellate *Karenia brevis*. The largest product illustrated, PbTx-2 (C_{50}), is known to arise by a complex polyketide pathway. The remarkable structural

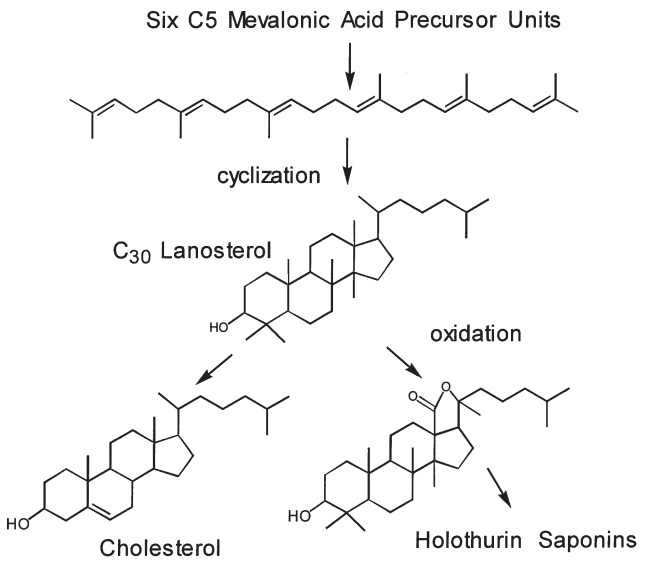


Figure 2 Lanosterol is the key intermediate in a convergent pathway. Normal processing yields primary lipid components such as cholesterol, while oxidation of a single methyl group leads to marine saponins.

similarity of the other two smaller polyethers such as brevenal (C_{39}) and hemibrevetoxin (C_{28}) is obvious and supports the premise that a similar biosynthetic pathway is followed in the production of these compounds. But which came first in the evolution of the cell? Although PbTx-2 was discovered first—due to its potent ichthyotoxic properties and respiratory effects on humans—the smaller non-toxic derivatives are clearly products of a shorter biosynthetic process that could have preceded the generation of the larger toxic metabolite.

The development of a biosynthetic pathway by a divergent process is well illustrated in the biosynthesis of the holothurin saponins. The established route to the isoprenoid-derived sterols is shown in Figure 2. A simple mutation in the pathway, involving oxidation of the C-19 methyl group to a carboxyl function, followed by cyclization to form a γ-lactone ring provides a new intermediate molecule that is the progenitor for many related saponins. Thus formation of this lactone intermediate immediately re-directs the biosynthetic flow towards a series of secondary metabolites and away from the primary sterol metabolites. Finally, a convergent process brings two biosynthetic pathways together to form a new product. For example, the fusion of two biosynthetic processes results in the formation of the microcystin group of blue-green algal toxins (Fig. 3). The ADDA function of the molecule—essential for activity—is derived from a polyketide (PK) pathway, while an NRPS complex is required in the assembly of the cyclic heptapeptide moiety. Fusion of these two enzyme processes results in the biosynthetic hybrids known as microcystins (Moffitt and Nielan, 2003). This hybridization of biosynthetic pathways is perhaps one of the most common routes to metabolite biodiversity in nature.

An example of the biological consequence of a conver-

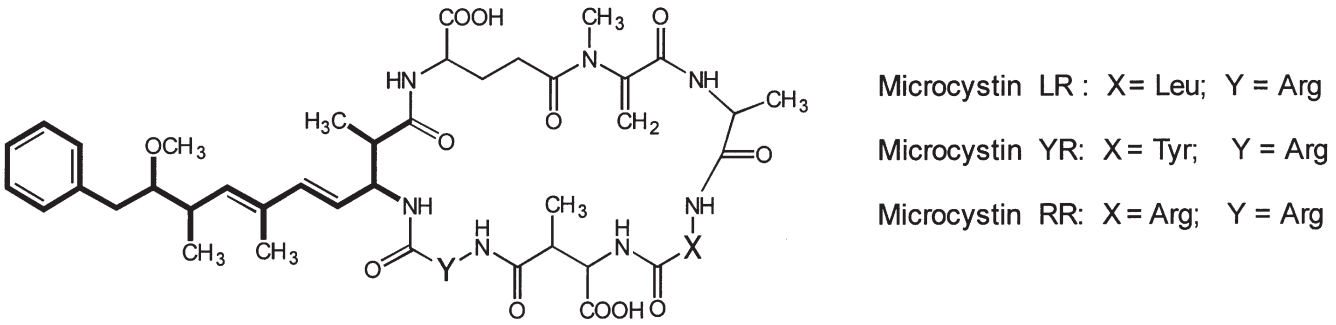
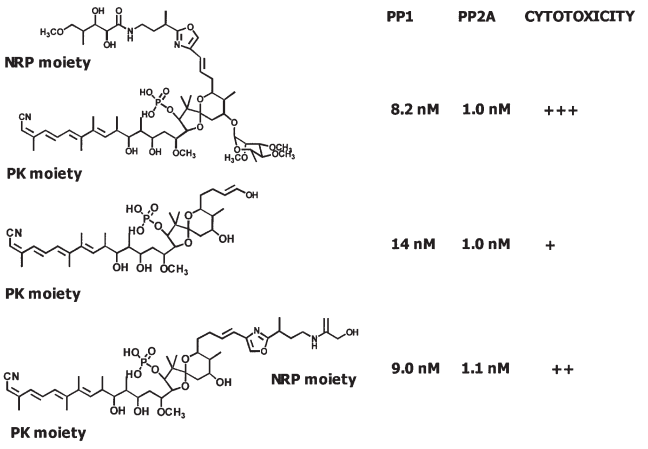


Figure 3 The microcystins are an example of a convergent biosynthetic pathway. The portion of the structure derived from a polyketide (PK) pathway is shown in bold, while the peptide portion is assembled from a non-ribosomal peptide synthesis (NRPS) pathway.

gent biosynthetic pathway is illustrated by the phosphatase inhibitor calyculin A (Fig. 4), isolated from extracts of the sponge *Discodermia calyx.* Calyculin A is derived by the fusion of a PK and NRPS pathway, and Fusetani and co-workers (Wakimoto *et al.,* 2002) examined the phosphatase inhibitory effects of a range of semi-synthetic products as shown in Figure 4. The complete parent molecule demonstrates remarkable potency. The derivative containing the peptide portion, but lacking the polyketide-derived moiety, is also active *in vitro* but less active *in vivo.* On the other hand, the polyketide-derived portion is much less active in either milieu. These results could be interpreted to mean that a combination product, the result of a convergent biosynthetic pathway, is best for cytotoxicity *and* penetration or transportation across the cell wall. Thus by linking the polyketide-derived portion with the peptide moiety, a more useful hybrid metabolite results.

Thus organisms lacking an immune system have evolved complex biosynthetic pathways leading to the generation of secondary metabolites. Many of these metabolites display powerful biological activity that can be useful in avoiding predation, or in capturing prey. Such an offensive



	PP1	PP2A	CYTOTOXICITY
	8.2 nM	1.0 nM	+++
	14 nM	1.0 nM	+
	9.0 nM	1.1 nM	++

Figure 4 The effect of different portions of the polyketide hybrid molecule Calyculin A on biological activity. Although the derivative containing only the peptide moiety is active *in vitro,* it lacks the overall potency *in vivo.* (Adapted from Wakimoto *et al.,* 2002).

role for a secondary metabolite is beautifully illustrated by the evolutionary development of muricid snails. These voracious carnivores acquire food by drilling a small hole in the shell of other shellfish, then inserting their proboscis through the hole to consume the contents of their prey. Snails are cannibalistic and to protect themselves from other carnivores, early species of snails adopted very thick and spiky shells as a mechanical means of defense. This was reasonably effective since the drilling process took days to accomplish. Over the course of evolutionary time, some snails acquired the ability to produce or biosynthesize a variety of simple choline esters (Fig. 5) that are effective muscle relaxants (Roseghini *et al.,* 1996). Release of these compounds in the vicinity of the prey causes the shell to open involuntarily, rendering the paralyzed shellfish an easy victim. This chemical approach to acquiring prey conferred an immediate advantage to the predator, and again, over the course of evolutionary time, this method of attack has been adopted by many species of muricid snails. Interestingly, they all still retain their shell-drilling ability, which they invoke when competition for prey is minimal, and there is more time to acquire food.

Cone snails provide another dramatic example of the use of metabolites in the capture of prey. Cone snails eject a harpoon-like device through which a range of neuropeptides can be injected. These peptides have the effect of instantly immobilizing, paralyzing, anesthetizing, and killing the prey. Thus a passing fish, when impaled by the harpoon, is immediately rendered helpless and is soon consumed by the snail. The suite of peptides required to effect this process is remarkable, and over 300 different peptides have been isolated from the venom of a single cone snail (Olivera and Cruz, 2001). Sea anemones are another group of marine organisms that effectively use peptides to capture prey. These are structurally different peptides from those used by cone snails, and in this case the mode of action is the formation

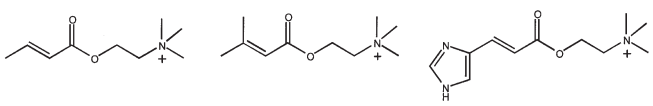


Figure 5 Choline esters produced by muricid snails as chemical agents to expedite the capture of prey.

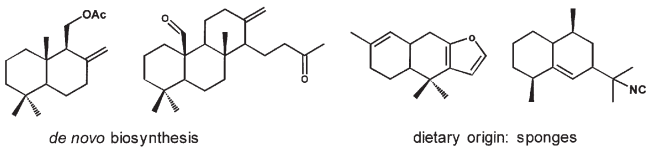


Figure 6 Examples of protective biosynthetic and dietary terpenoids found in the nudibranch *Cadlina luteomarginata.*

of pores in choline and sphingomyelin membranes (Anderluh and Macek, 2002).

Peptides can also play a defensive role in many organisms (Mebs, 2001). Indeed, peptides are considered by many to be the most ancient of antimicrobial agents (Zasloff, 2002) and are found in a broad range of organisms including mammals, fish and bacteria. Present on the surface of many organisms, they are considered to be the primary line of defense against invading organisms. These peptides contain a high proportion of sulfur-containing amino acids such as cysteine, and their 3-D structure and conformation arise by the formation of internal sulfide bridges that render them resistant to many common peptidases. The amphipathic organization of the positively charged and hydrophobic regions of these peptides is a key feature of their biological activity, permitting them to bind effectively to negatively charged membranes. Regardless of the organism in question, all these peptides are produced constitutively, always there to capture prey should it pass close by, or prevent attack by an opportunistic predator.

Some organisms that produce neither constitutive nor defensive compounds acquire defensive compounds through their diet. The slow-moving nudibranch *Cadlina luteomarginata* possesses no protective shell yet survives comfortably in a competitive environment. Andersen and co-workers (Dumdei *et al.,* 1997) have identified over 30 terpenoid structures in the skin, mucus, and egg masses of this mollusk (Fig. 6). Many of these compounds are of dietary origin and are found in a sponge the mollusk frequently consumes. These metabolites are cytotoxic and possess anti-feedant properties, and presumably these same properties that protect the sponge are also utilized by the mollusk. Interestingly, as these mollusks have evolved, they have developed the ability to synthesize many of these terpenes *in vivo,* thus they do not have to rely solely on their diet for these defensive compounds (Kubanek *et al.,* 1997).

Other defensive strategies are to immobilize or bind the toxin in such a way that it is rendered harmless. The saxiphilin proteins found in mussels exposed to the paralytic shellfish poisoning (PSP) toxins serve such a role (Lewellyn and Moczydlowski, 1994). These transferrin-like proteins bind the guanadine group of PSP toxins, and a number of such proteins have been identified from different species. Yet another defensive strategy is to develop immunity from toxins by altering the structure of the target molecule in the prey organism. Now the toxin is no longer effective and, as in the newt or nudibranch, even permits them to safely store the toxin, thus ensuring no other predator will attack. The target of PSP and TTX toxins is site 1 of the Na channel. Through a simple mutation of the ion channel, often only the interchange of a single amino acid (White *et al.,* 1998; Yotsu-Yamashita *et al.,* 2000) renders the protein immune to the effects of these toxins.

The idea of chemical immunity and storage of toxins by a potential prey organism such as the newt or nudibranch raises the question as to how toxin-producing organisms themselves find protection from the very toxins they produce. This was a question posed very early by researchers studying the production of antibiotics by bacteria—how do the bacteria avoid killing themselves? Studies have revealed a series of strategies to avoid self-destruction (Walsh, 2000) and some specific examples are illustrated in Fig. 7. Site-specific phosphorylation of streptomycin renders the antibiotic harmless within the cell, and the phosphate groups are selectively removed upon excretion of the antibiotic to the outside world. Erythromycin binds to 23S RNA, shutting down protein synthesis, but in the producing organism *Saccharopolyspora erythraea,* methylation of the adenosine at position 2058 in the 23S RNA precludes any such binding from occurring. The toxicity of mitomycin results from its conversion to the extremely reactive quinone methide. In the producing organism, this conversion is prevented by an enzyme that instantly reverses the process, while the same protein apparently acts as a binding protein to provide an additional self-protection strategy (He *et al.,* 2001).

A self-defense strategy does not seem necessary for many marine toxins such as PSP, tetrodotoxin or the brevetoxins, since their only biological targets appear to be higher order species such as animals with a central nervous system.

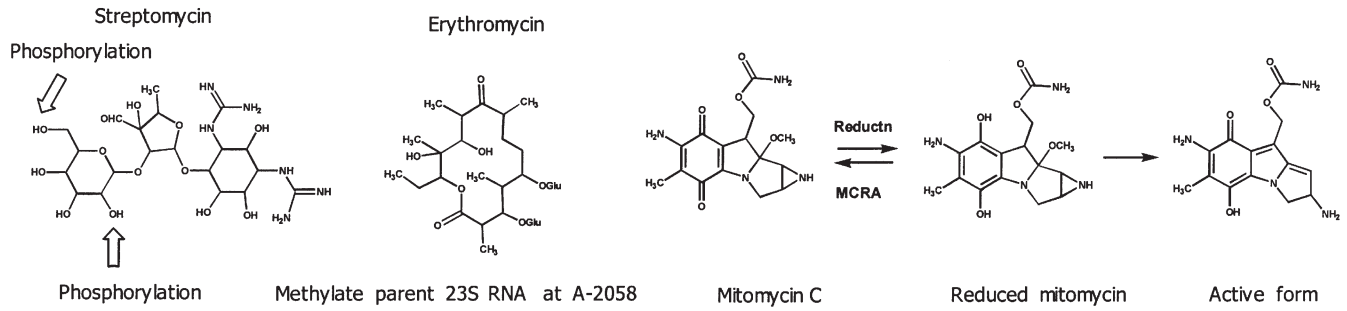


Figure 7 Some strategies adopted by organisms to gain self-protection from the bioactive compounds they produce. Streptomycin (product modification), erythromycin (target modification) and mitomycin C (product activation).

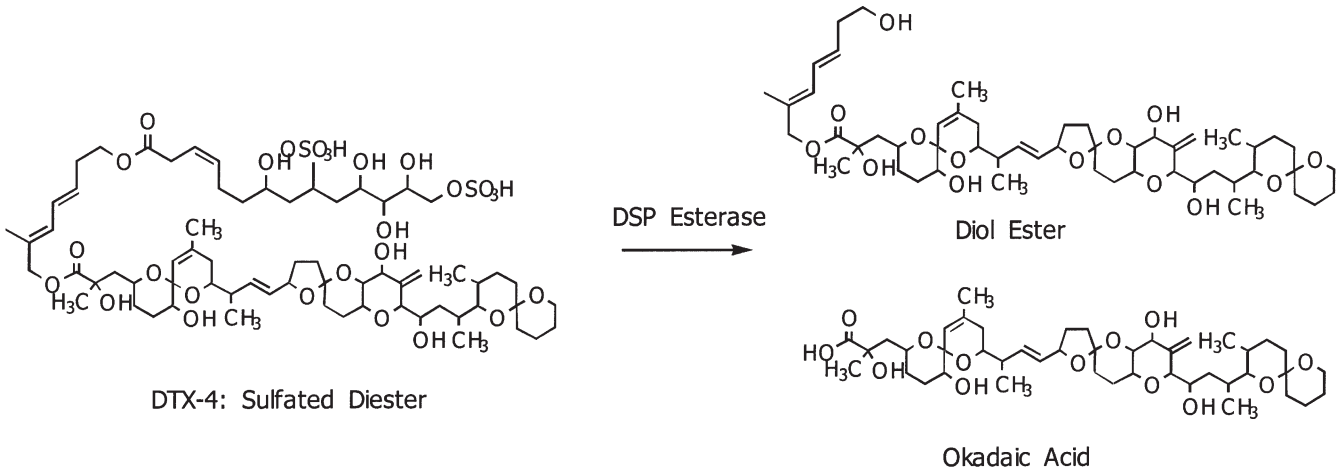


Figure 8 Scheme illustrating the storage of DSP toxins as non-toxic sulfated diesters in *P. lima* cells, and their conversion to active components upon enzyme catalyzed hydrolysis by a putative DSP esterase.

However, this is not the case with the DSP toxins okadaic acid and DTX-1, which inhibit the eukaryotic serine/threonine phosphatases PP1 and PP2A—key enzymes in many primary metabolic functions. These phosphatases are present in the DSP producer *Prorocentrum lima,* and are susceptible to the DSP toxins *in vitro,* yet *in vivo,* the organism is unaffected by their production or presence. The discovery that the large sulfated diesters such as DTX-4 are the initial products of DSP toxin biosynthesis in the organism offered an explanation (Hu *et al.,* 1995; Needham *et al.,* 1995; MacPherson *et al.,* 2003). These sulfate esters are not phosphatase inhibitors, and it is proposed that the toxins are stored and transported within the cell in this form, only to be converted to the toxin form by a specific esterase located in the cell membrane or cell surface. Some support for this comes from the observation that rupture of the cells by freeze-thawing prior to extraction results in recovery of the free acid form principally, whereas immediate extraction of fresh cells (Hu *et al.,* 1995; Williams and Wright unpublished data) yields mainly esters. From these results it is possible to speculate that attack or rupture of DSP-producing *Prorocentrum* cells would expose the sulfated diesters to esterases, thus releasing the free toxin and deterring further attack.

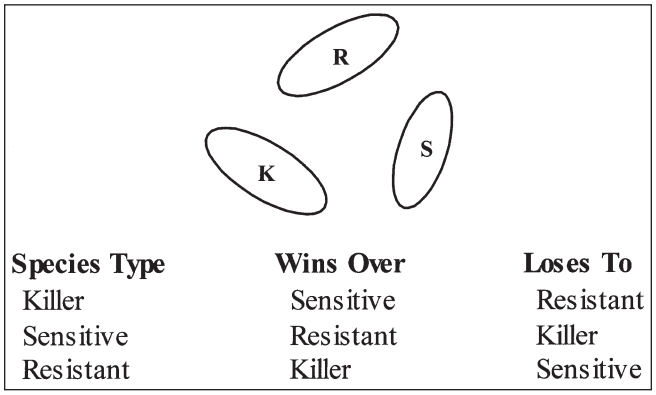


Figure 9 Rock, Paper, Scissors Model. In the biological world, three species exist: Killer (K), Sensitive (S), and Resistant (R). (Adapted from Kerr *et al.,* 2002).

While it is straightforward to make the conceptual case that production of toxins or acquisition of immunity from toxins contributes to the fitness of an organism (Wright and Cembella, 1998), there are many species in the ocean that do not appear to make toxins yet survive successfully in this competitive milieu. For example, several species of *Prorocentrum* do not biosynthesize DSP toxins, and variable toxicity of strains within a unialgal phytoplankton bloom is well recognized (Bravo *et al.,* 2001). A biological model (Fig. 9) based on the children's game of "rock-paper-scissors" (RPS) elegantly predicts increased biodiversity as a result of chemical warfare between microbes (Kennedy *et al.,* 2002; Kerr *et al.,* 2002), and may provide an answer to this "paradox of the plankton," as it has been described (Czaran *et al.,* 2002; Frean and Abraham, 2001). This mathematical analysis of diversity based on the RPS game considers three competing species: Killer (scissors), Sensitive (paper) and Resistant (rock). In this biological version, the killer strain must carry a metabolic and energy penalty for the production of, and immunity to, the toxin it produces. The resistant strain also carries a metabolic penalty, in possessing one or more resistance factors to the toxin, but this is not as great as that carried by the killer strain. Finally, the sensitive strain carries no such metabolic or energy penalties and can grow or multiply more rapidly then either of the other two strains.

In the case of bacteria growing in close proximity, the rock-paper-scissors model has been demonstrated to increase diversity, rather than hinder it, and could explain the variety of toxic clones that are observed in a phytoplankton bloom. Thus while toxin-producers appear to have an advantage in terms of survival, when growing conditions are less than optimal, the sensitive, faster-growing strain will be more successful.

In summary, as more secondary metabolites are identified and biological activities can be assigned to them, it

becomes possible to define a role for some of these compounds in maintaining or increasing the fitness of the producing species. Nevertheless, a considerable metabolic or energy penalty results from the biosynthesis of complex bioactive compounds, which are often the result of elaborate biosynthetic pathways. The picture is further complicated by the acquisition of bioactive compounds through diet, by commensal or symbiotic relationships, or by the lateral transfer of the biosynthetic genes from one species to another. While it may be possible to propose a beneficial role for such metabolites in the survival of the organism, the apparently successful survival of species that produce no such bioactive compounds provides an interesting paradox that may be explained by consideration of the rock-paper-scissors model. This model, which can be applied to a variety of biological and physical systems, elegantly explains the successful co-existence of toxic and non-toxic species in phytoplankton blooms.

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ECOLOGY SESSIONS

The Potential Use of a Hydrodynamic Model in the Prediction of Harmful Algal Blooms in the Southern Benguela

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Abstract

Advection is considered an important source of variation in planktonic ecosystems. At the event scale (3–6 days), the population dynamics model of harmful blooms, particularly in local areas of potential impact, may be reduced to the advective term. At this scale of interest, hydrodynamic models are likely to be very useful in predicting the timing, location and duration of blooms. The value of a hydrodynamic model in establishing the spatial development and advection of red tide responsible for an estimated mortality of 1200 tons of rock lobster in the Elands Bay–St. Helena Bay region in 2002 is assessed.

Introduction

The analysis of spatial and temporal patterns in planktonic ecosystems differs fundamentally from that in benthic or terrestrial ecosystems because, by definition, planktonic organisms are passively transported by the medium in which they exist. In stratified coastal waters of the southern Benguela, harmful blooms of motile algae have appeared far more rapidly and to reach concentrations far higher than can be explained by local growth. For this reason, advecton has been considered an important source of variation with time. It is important not to interpret a rapid increase in local concentration as the rapid growth of a local population, when it is the result of advection from elsewhere. It is therefore important to examine these blooms from a quantitative population dynamics perspective that includes not only the population in the region of potential impact, but also the dynamics of the broader population from which they may originate. In some cases it is appropriate to consider the local population in the region of interest as an integral part of a much larger population, and in others it is useful to consider the population as being of limited spatial extent and to examine the advection of that population in a Lagrangian manner (Donaghay and Osborn 1997).

This paper assesses the value of a hydrodynamic model in establishing the spatial development and advection of

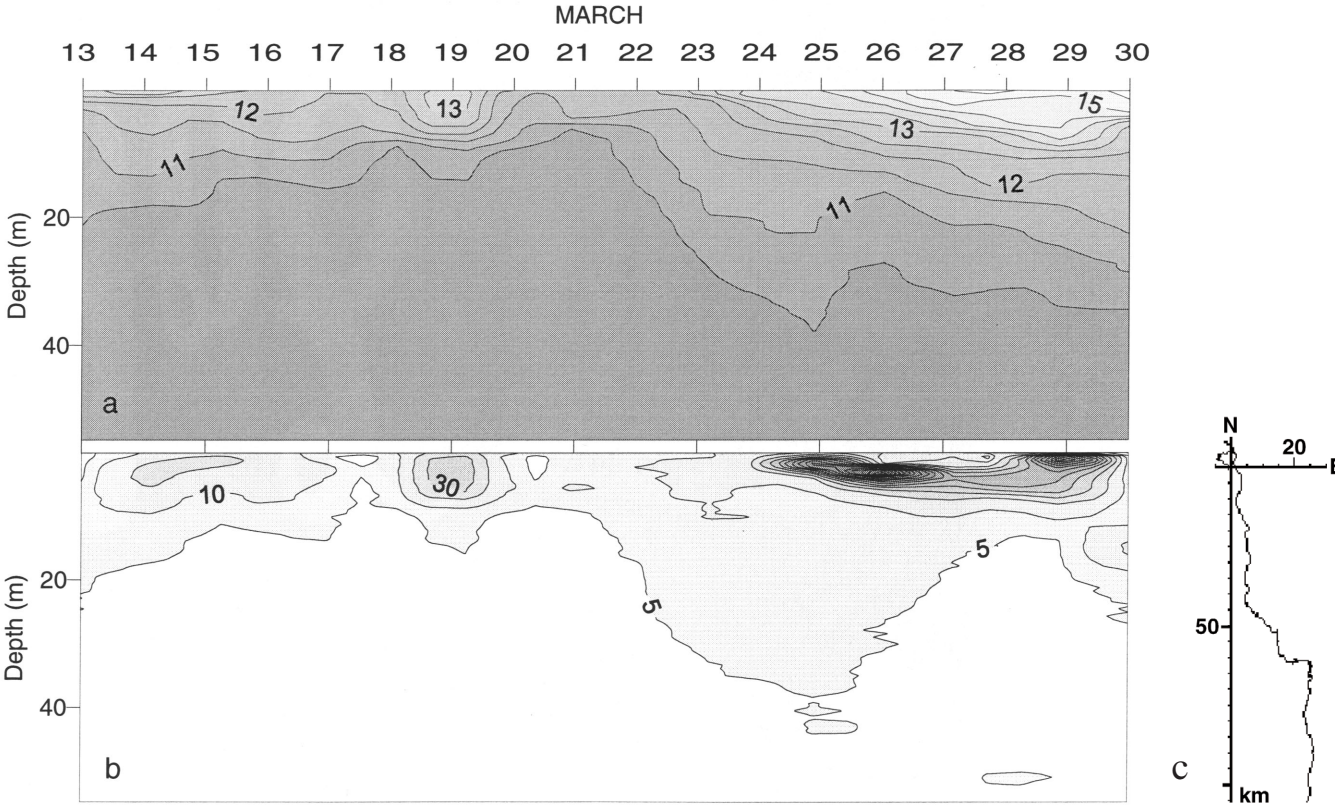

Figure 1 Time series 13–30 March 2001 of **a**, temperature (°C); **b**, chlorophyll *a* (mg m^{-3}); and **c**, surface currents [progressive vector] off Lambert's Bay on the Namaqua shelf.

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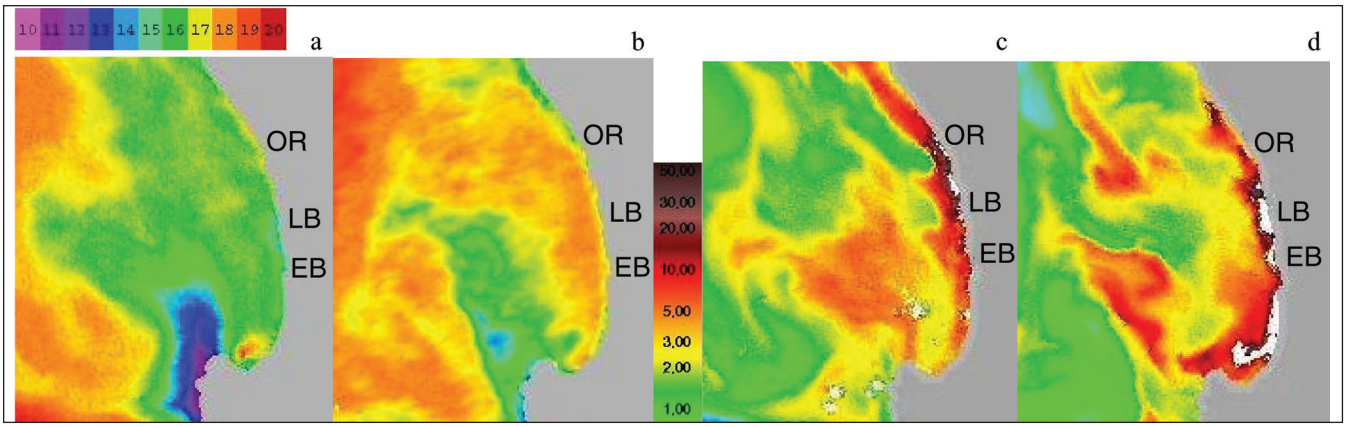

Figure 2 Advanced Very High Resolution Radiometer (AVHRR) observations of Sea Surface Temperature (SST, °C) on (**a**) 24 and (**b**) 30 January, 2002; and chl *a* (mg m^{-3}) distributions as observed by SeaWiFs (Source: S. Weeks of Ocean Space) on (**c**) 24 and (**d**) 31 January, 2002. In the greater St. Helena Bay region, the whitened areas on the SeaWiFs image on 31 January 2002 are masked due to very high chl *a* concentrations and consequent saturation of the nLw bands.

blooms in the region between the Namaqua and Cape Columbine upwelling cells, an area particularly susceptible to red tide formation and its negative impacts. A historical perspective of faunal mortalities associated with red tide in this region is given by Cockcroft (2000), which documents the vulnerability of the Elands Bay–St. Helena Bay area to mortalities associated with low oxygen events following the degradation of high biomass dinoflagellate blooms. These blooms are easily detected by means of satellite observations of ocean colour, but hydrodynamic models are required in order to predict when and where these events may be of potential significance. Our model is used specifically to investigate the advection of red tide into the Elands Bay–St. Helena Bay region in January 2002, the decay of which caused an estimated mortality of 1200 tons of rock lobster.

Field Observations of Bloom Advection

Several field studies have documented the hydrographic conditions favouring the development and transport of red tide over the southern Namaqua shelf (Pitcher *et al.,* 1998; Probyn *et al.,* 2000). In particular, recent studies incorporating the simultaneous measurement of currents with observations of blooms, have enabled the cross-shelf and alongshore currents associated with the advection of red tide to be quantified (Pitcher *et al.,* submitted). Typically, dinoflagellate blooms are associated with frontal systems demarcating an inshore band of upwelling. Here the surface drift is mainly northerly, but wind reversals cause this frontal system and associated bloom to move shoreward. The development of an inshore countercurrent results in the southward propagation of blooms.

A time series of vertical profiles of temperature and chlorophyll *a*, and current measurements (Fig. 1, adapted from Pitcher *et al.,* submitted) at an inshore station on the southern Namaqua shelf provided a useful account of the current flow during the development of red tide inshore. This time series demonstrated the development of red tide (25–29 March 2001) at the site of monitoring following the reversal of upwelling winds. Progressive current vectors at the surface indicated that flow was predominantly southward, introducing red tide (>100 mg chl *a* m^{-3}) into the region during the latter half of the time series. Although southward flow dominated, a period of onshore flow, coinciding with the reversal of upwelling winds, introduced the bloom to the coastal environment. This period of onshore flow separated diatom domination of the inshore population during the first half of the time series from dinoflagellate domination of the phytoplankton during the second half of the time series. The insight generated by these studies into the origin and transport of blooms allow the use of hydrodynamic models to predict the timing and duration of coastal blooms at any single location.

Model Description

A 3D hydrodynamic model was developed for the summer of January 2002, covering an area of the southern Namaqua shelf sufficiently large to include important circulation patterns. Modelling the hydrodynamics of this area was undertaken using the DELFT3D-FLOW model. A 10-layer sigma curvilinear grid was designed to follow the shoreline. The grid had 130 × 61 lines and was designed so that the cell size at the offshore boundary was as large as 8 km × 11 km, but was refined to a size of approximately 60 × 100 m inshore. The grid had 3202 active cells and covered a distance of approximately 240 km alongshore and 120 km cross-shore. A detailed bathymetry of the model domain was compiled by combining survey data with hydrographic chart data. To avoid numerical instability, the depths offshore were limited to 330 m. At the open boundaries, a water level time series was specified based on the predicted tide. The eight largest amplitude tidal constituents along the West Coast were applied to predict the tide (Rozenthal and Grant, 1989), which was specified at 10-minute intervals. The wind data applied in the simulations was measured hourly at St. Helena Bay. The

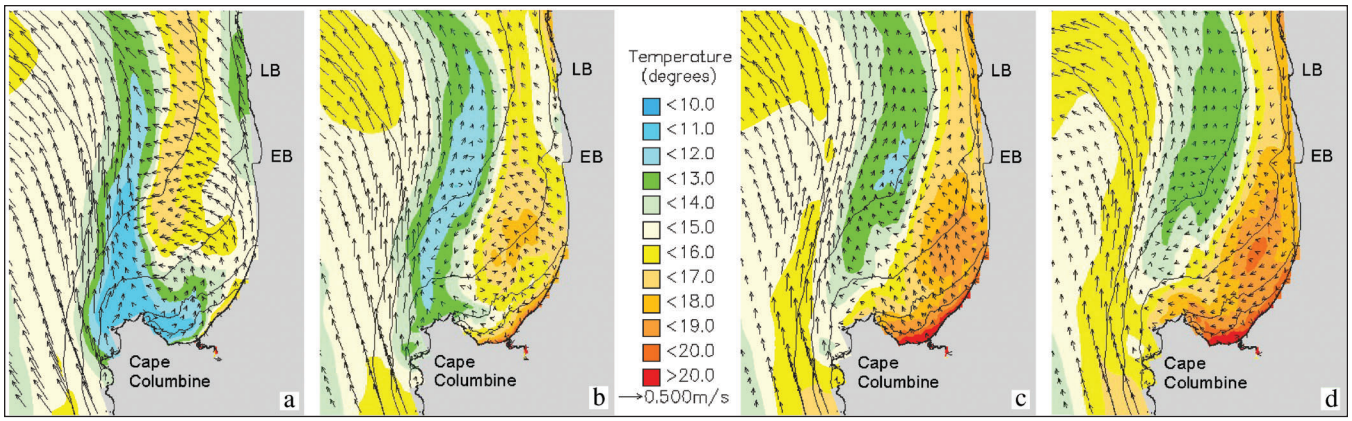

Figure 3 Model output of SST and current vectors at (**a**) 00h00 on 25 January 2002, (**b**) 00h00 on 27 January 2002, (**c**) 21h00 on 28 January 2002, and (**d**) 00h00 on 30 January 2002 (LB–Lambert's Bay; EB–Elands Bay).

atmospheric measurements applied in the simulations included air temperature, relative humidity and cloud cover data as measured at Cape Columbine. Temperature was modelled by applying static boundary conditions at the three open boundaries. A time step of 2 minutes was applied and all model runs were initiated with zero velocity. The initial sea level was set to a constant according to the initial tidal sea level specified at the open boundaries.

Model Output and Satellite-Derived Observations of SST and Ocean Colour

Satellite observations on 24 January 2002 depicted conditions following a period of moderate upwelling (Fig. 2a). A distinct plume of upwelled water was evident off Cape Columbine and cooler water was also present inshore off Lambert's Bay and Elands Bay. Observations of ocean colour at this time indicated the presence of a bloom designated by moderately high chlorophyll concentrations (<50 mg m^{-3}) centered off the Olifants River (Fig. 2c). Chlorophyll concentrations in the Elands Bay–St. Helena Bay area were generally <10 mg m^{-3}. Output from the hydrodynamic model on 25 January 2002 accurately portrayed the hydrographic conditions, depicting the coldwater plume off Cape Columbine and the narrow band of upwelling off Lambert's Bay and Elands Bay (Fig. 3a). Current vectors depicted offshore flow in association with the narrow band of upwelling inshore and northerly flow in association with the Cape Columbine upwelling plume. Winds favouring upwelling abated on 25 January 2002 and a quiescent period of several days followed. Model output during this period clearly demonstrated the hydrographic features important in the shoreward accumulation and southward transport of red tide (Fig. 3b,c,d). The role of the Cape Columbine plume in establishing an area of retention between the plume and coast was evident. There was development of weak onshore flow and the establishment of an inshore counter current introducing warmer water from the north. Under these conditions it was expected that bloom concentrations would increase inshore and that the bloom would be transported southward. Satellite observations of seas surface temperature (SST) on 30 January, and of ocean color on 31 January 2002, confirm these expectations. The intensity of upwelling off Cape Columbine declined and water within the Elands Bay–St. Helena Bay region warmed considerably (Fig. 2b). Most important was the development of a very high biomass bloom extending from Donkin Bay into St. Helena Bay (Fig. 2d).

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Four *Karenia brevis* Blooms: A Comparative Analysis

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Abstract

Four major toxic *Karenia brevis* (= *Gymnodinium breve*) blooms occurred on the West Florida Shelf between October 1998 and January 2002. Population abundance ranged from one thousand to 5.4 million cells/liter. Similarities in the hydrographic and nutrient regime, and physiological indicators were examined to assess maintenance mechanisms. All blooms appeared after breakdown of vertical stratification, with three of the blooms associated with nearshore thermal or salinity fronts after onshore transport. Water column DIN:DIP molar ratios were generally less than 1.0. Cellular molar C:N ratios were near Redfield but molar C:P, N:P, and the weight ratio of P:Chl all suggested P-deficiency. Elevated dissolved silicate concentrations at the start of each bloom indicated estuarine flux of water and nutrients into coastal waters. Calculations of TN and TP flux from Tampa Bay and Charlotte Harbor suggest that estuarine flux would supply a highly variable fraction of the N and P required for a moderate population (3×10^5 cells/liter) growing at 0.2 divisions per day. A re-evaluation of nutrient sources to support blooms $>10^5$ cells/liter is needed.

Introduction

Red tides caused by the toxic dinoflagellate *Karenia brevis* on the West Florida Shelf originate in oligotrophic offshore waters (Steidinger and Haddad, 1981) with subsequent transport via winds and tidal currents (Steidinger and Haddad, 1981). Thermal fronts can act both as a barrier and transport mechanism and as a concentrating mechanism for normally disparate populations of *K. brevis* in shelf waters. The result is a "bloom" which originated from a concentration process rather than from enhanced growth rates. Primary production rates within these blooms are 2–3 fold higher than background rates during non-bloom periods, primarily because of elevated biomass in large patches (Vargo *et al.,* 1987). However, nutrient (inorganic nitrogen and phosphate) levels remain unchanged and reflect the oligotrophic nature of the West Florida Shelf (Heil *et al.,* 2001).

The sources of major nutrients required to maintain blooms that persist for several months has not been identified (Steidinger *et al.,* 1998, Vargo *et al.,* 2001). Hypotheses range from nitrogen input from N-fixation during *Trichodesmium* blooms (Lenes *et al.,* 2001; Walsh and Steidinger (2001), remineralization of near bottom diatom blooms fueled by shelf-break upwelling (Walsh *et al.,* 2003) and estuarine flux of N and P when blooms reach coastal waters. We examined four *Karenia brevis* blooms that occurred during the ECOHAB:Florida program on the West Florida Shelf to assess potential nutrient sources for initiation and maintenance mechanisms.

Methods

Methods for all parameters mentioned can be found in Vargo *et al.* (2001) and Heil *et al.* (2001).

Results and Discussion

Four major toxic *Karenia brevis* blooms occurred on the West Florida Shelf between October 1998 and January 2002 (Table 1). Population levels in each bloom ranged from approximately one to 5.4 million cells/liter. The spatial extent of each bloom varied from hundreds to thousands of square kilometers. Water column dissolved inorganic nitrogen to dissolved inorganic phosphorus molar ratios (DIN:DIP) were generally less than 1.0, suggesting N-limitation paralleling P-enrichment from nearby rivers which drain commercially exploited phosphate deposits. Elevated dissolved silicate concentrations were associated with high cell abundance for all blooms. High silicate levels typically found in local estuaries are indicative of transport of es-

Table 1 Summary of four *Karenia brevis* blooms in the ECOHAB:Florida area.

		Maximum Conditions (First Month of Bloom)				
Year	Duration (Months)	Cells/L $\times 10^6$	Chl *a* µg/L km^2	Area Mean (Range)	Dissol. Molar N:P Mean (Range)	Silicate (µM)
1998–1999	4	1.12	4.77	667	0.03 (0–0.03)	2.73 (1.7–4.6)
1999–2000	6	5.35	27.10	4000	0.79 (0.1–2.3)	8.78 (1.5–19.2)
2000a*	2	2.93	5.50	6300	0.47 (0.1–0.2)	2.49 (0.1–11.8)
2000b*	2	0.77	4.50	1000	0.81 (0.1–1.7)	2.05 (0.4–7.5)
2001–2002	10	11.23	43.28	7120	0.92 (0.08–2.4)	5.21 (2.4–9.2)

*Blooms were spatially separate.

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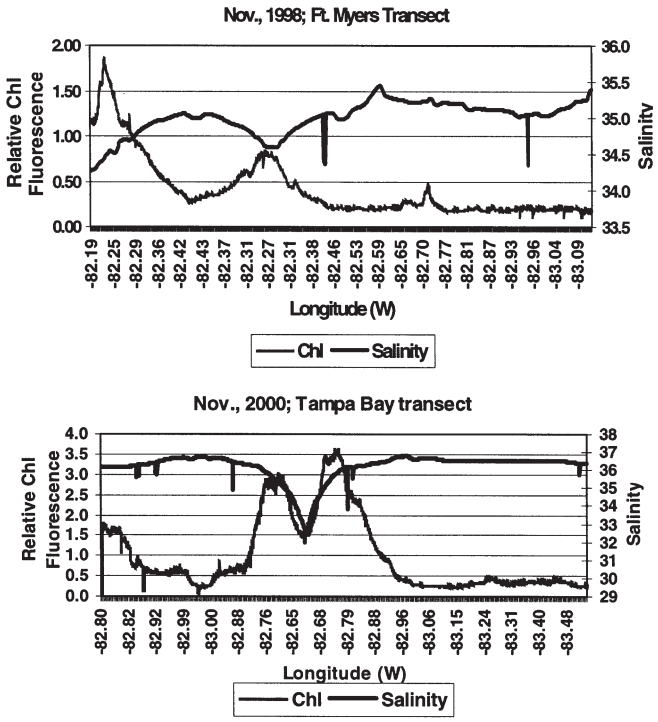

Figure 1 Examples of the relationship between blooms and salinity fronts.

tuarine water into the coastal zone and the potential export of N and P for bloom maintenance.

Despite the differences in magnitude and areal extent, there were several features in the hydrographic and nutrient regime that were common to each bloom. All blooms appeared after breakdown of vertical stratification (Vargo *et al.,* 2001). Breakdown of stratification is likely due to a combination of wind mixing and onshore transport of water as a result of upwelling favorable winds. Transport of estuarine water into the coastal zone leads to horizontal rather than vertical stratification and the development of thermal and salinity fronts. All four blooms were associated with near-shore thermal or salinity fronts as indicated by underway salinity and *in vivo* chlorophyll fluorescence measurements (Fig. 1).

Cellular indicators of physiological state varied between blooms and with biomass levels. Typical molar ratios for cultures of *K. brevis* are as follows: C:N = 6–11; C:P = 90–107; N:P = 9–19 (Heil, 1986). Similarly, a range of typical C:Chl, N:Chl, and P:Chl weight ratios for *K. brevis* cultures are as follows: C:Chl = 241–516; N:Chl = 21–79; P:Chl = 6–15 (Heil, 1986). As noted above, dissolved inorganic N:P ratios indicate phosphorus enrichment relative to N availability. Particulate C:N molar ratios for three of the blooms were near Redfield (5–7), indicative of nutrient sufficient populations, while values of <1.0 were found for populations greater than 5 million cells/liter during the 1999–2000 bloom, suggesting N-enrichment (Table 2). However, particulate molar C:P and N:P ratios were high and the weight ratios of P:Chl for all of the blooms were low and indicative of P-limitation (Table 2). Finding P limitation in nearshore *K. brevis* blooms is somewhat enigmatic, given the presence of inland phosphate deposits. However, Vargo and Shanley (1985) described P-limited blooms within Charlotte Harbor, a highly P-enriched estuary. The overriding issue is: How can high biomass blooms be maintained for months when in nearshore waters? Since blooms are associated with coastal salinity and temperature fronts, an obvious source should be estuarine flux.

We used several methods to estimate the flux of nutrients into coastal waters from the two primary estuaries on the west central Florida coast (Table 3): 1. Daily fresh water flows for Tampa Bay and Charlotte Harbor were totaled and multiplied by the concentration of TN and TP measured in the region nearest the mouth of each estuary, with nutrient flux estimated by assuming that flow out was essentially equivalent to flow in (but we recognize that the flow could be underestimated because of stream gauge location and nutrient values are spatially variable); 2. the WASP model as used by Martin *et al.* (1996) to calculate fluxes between Tampa Bay segments for N only; 3. the value from Ross (1973) who calculated that 25.8% of the tidal quantity of water is permanently removed from Tampa Bay by mixing with Gulf of Mexico water; and 4. a simulation using a 3-D model which incorporated freshwater flows and the tidal excursion (Weisberg, pers. comm).

Estimates of TN and TP fluxes from Tampa Bay based on these methods indicated that estuarine transport could supply from 5% to 20% of required N and from 4% to 90% of required P for the daily growth needs of a moderate population (3×10^5 cells/liter) of *Karenia brevis* (Table 4).

Table 2 Summary of physiological indicators for the first month or population maximum of four *Karenia brevis* blooms.

	Duration	Chl *a*	Range of Particulate Molar Ratios			Range of Particulate Weight Ratios		
Year	(Months)	(μg/L)	C:N	C:P	N:P	C:Chl	N:Chl	P:Chl
1998–1999	4	4.77	4–18	170–820	21–80	84–390	16–55	0.9–1.6
1999–2000	6	27.10	0.4–5.5	15–174	23–167	7.5–91	11–124	0.6–1.4
2000a*	2	5.50	4–19	188–906	30–62	174–1022	34–60	1.3–4.4
2000b*	2	4.50	1–2.3	475–1149	461–489	432–1776	489–883	1.4–3.9
2001–2002	10	43.28	4–10	316–987	65–115	56–180	11–33	0.4–0.8

*Blooms were spatially separate.

Table 3 Calculated TN and TP estuarine flux from Tampa Bay and Charlotte Harbor.

Method	Total Flux (moles/day * 10^5) TN	TP	Flux Diluted into Control Vol. (µg-at/L/d) TN	TP
Freshwater flow[1]				
Nov 87–Feb 99	1.13	0.0070	0.0042	0.00030
Oct 99–Mar 00	1.69	0.0053	0.0053	0.00022
Oct/Nov 00	0.73	0.0018	0.0027	0.00007
EPA WASP[2]	2.44		0.0062	
Fractional Exchange[3]	21.3	3.68	0.054	0.011
ECOHAB Model[4]	10.10	0.75	0.029	0.0022

[1]Dixon, pers. comm. (bloom periods). [2]Martin *et al.,* 1996 (annual average). [3]Ross, 1973 (annual average). [4]Weisberg *et al.,* pers. comm. (spring 1998)

Therefore, estuarine fluxes of N and P are barely sufficient to support moderate populations in near-shore waters. Most blooms, however, have population levels greater than 3×10^5 cells/liter and persist for months (see Table 1). Supplying nutrients for bloom maintenance is a major dilemma. Use of N from other nitrogen sources noted above may contribute sufficient quantities to maintain growth, but processes such as N-fixation also require P and therefore exacerbate the potential for P-deficiency. Other than estuarine flux, no other sources of P have been identified. One possible mechanism may involve vertical migratory activity that allows dense surface populations to form during the light period (>10^6 cells/liter). These dense populations then disperse throughout the water column during darkness (Heil, 1986). We speculate that this dispersal might allow cells to utilize available nutrients from the entire water column and achieve nutrient sufficiency. This dilemma is being addressed and will be resolved when model estimates of estuarine N and P flux are complete.

Acknowledgements

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Table 4 The range of TN and TP required for growth of *K. brevis* at 0.2 divisions/day, the range of TN, TP flux diluted to the 10 m isobath and the % of requirements met by this flux from both Tampa Bay and Charlotte Harbor.

		Range		
		Required (µM d^{-1})	Flux (µM d^{-1})	% Supplied
TN	(low)	0.056	0.003	5.4
	(high)	0.267	0.054	20.2
TP	(low)	0.002	0.00007	3.5
	(high)	0.012	0.011	90.9

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Alexandrium fundyense Migration Patterns in the Bay of Fundy, Eastern Canada

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Abstract

Populations of *Alexandrium fundyense* were studied for diel vertical migration patterns at 2 h intervals during 30- and 22-h periods during July 2001 in the offshore waters of the Bay of Fundy, eastern Canada. Samples for phytoplankton and nutrients were collected at the surface and depths of 5, 10, 20, 30, 50 and 90 m. A CTD profiler was deployed for measurements of temperature, salinity, oxygen and fluorescence. Results suggest that *A. fundyense* vegetative cells, planozygotes and duplets concentrate in the upper waters, with highest concentrations observed most often in surface samples and concentrations decreasing significantly with depth. Highest concentrations were detected at 1230 h. No obvious vertical migration behaviour was detected. CTD data indicated that the water column was weakly stratified throughout the sampling period. Nutrient levels suggest that values in surface waters were reduced.

Introduction

Alexandrium fundyense, the organism responsible for paralytic shellfish poisoning (PSP), blooms annually in the Bay of Fundy generally during the months of May–August. Understanding features and mechanisms underlying the population dynamics of PSP producers such as *A. fundyense* is important to prediction of movement, occurrence, toxicity, environmental effects and ultimately to management of harmful algal blooms (HABs) and their impacts. Field and laboratory observations of dinoflagellates such as *A. tamarense, Heterocapsa* spp., *Karenia brevis* and *Dinophysis acuminata* show surface aggregations during the daytime and dispersal or diel vertical migration (DVM) at night (Seliger *et al.,* 1979; Olli, 1999). Some dinoflagellates migrate in order to utilize the deeper nitrate reserves for bloom development or respond to light and gravity. Others, in regions with a steep thermocline, may migrate to an environment suitable for bloom formation (MacIntyre *et al.,* 1997; Kamykowski *et al.,* 1998). The present study focuses on two surveys conducted in the offshore Bay of Fundy waters in July 2001 in which 30- and 22-h time series of the vertical distribution of *A. fundyense* cells and hydrographic conditions were obtained. The study was conducted during the end of the seasonal bloom of *A. fundyense.*

Materials and Methods

Water samples were collected at 2 h intervals for 30- and 22-h at depths of 0 (surface), 5, 10, 20 or 25, 30, 50 and 90 m on July 12/13 and July 16/17, 2001 at an offshore Bay of Fundy station (44°53′N–66°26′W). Total depth at the sampling station was approximately 100 m. *A. fundyense* cells were preserved in formalin:acetic acid and enumerated microscopically following settlement of 50 mL, using the Utermöhl technique. *A. fundyense* was confirmed by SEM and cells were counted as total cell number (per liter) as well as differentiated according to life cycle stages—asexual (vegetative: single, duplet, triplet, quadruplet) and sexual (fusing, planozygote, hypnozygote or resting cyst). Vertical depth profiles of temperature, salinity, density and fluorescence were obtained using a Seabird CTD profiler equipped with a YSI DO sensor and a WetLabs WETStar fluorometer. Nutrients (nitrate, nitrite, silicate, phosphate and ammonia) from the surface, 10 and 50 m were measured with a Technicon Autoanalyzer II.

Results and Discussion

Highest concentrations of *A. fundyense* throughout the 30- and 22-h surveys were observed in the upper 10 m of the water column in the surface mixed layer and pycnocline (Figs. 1–3). Fig. 1 shows examples of contour plots of the temporal variation in the vertical distribution of water density, total abundance, and fluorescence. Figs. 2 and 3 show examples of vertical depth profiles of total *A. fundyense* abundance and its life cycle stages (duplets, planozygotes and cysts), in addition to temperature, salinity, water density, and fluorescence. Fig. 2 shows daytime (1500 h on July 12) and nighttime (0300 July 13) profiles. Fig. 3 illustrates profiles from day (1430 h on July 16) and night (0430 h on July 17). Maximum cell densities were generally observed in surface samples, with the greatest concentration (2.74×10^5 cells $\cdot$ L^{-1}) observed at 1230 h on July 16. There was no detectable temporal pattern or difference between the day and night in the vertical distribution of *A. fundyense* cells with the sampling intervals and frequency used (Fig.1–3). This appears to be consistent with *A. fundyense* not exhibiting diurnal vertical migration. In contrast, field studies of *Alexandrium tamarense* show cells tending to either concentrate in the upper layers during the daytime in Hiroshima Bay (Itakura *et al.,* 2002), and in subsurface layers (3–15 m) under stratified conditions in the St. Lawrence Estuary, eastern Canada (Cembella and Therriault, 1989; Laroque and Cembella, 1990). Most studies on diel vertical migration have been done in the laboratory and show nocturnal migration of *A. tamarense* to the nitracline when the surface-layer nitrate has been depleted (MacIntyre *et al.,* 1997).

Although cysts were often observed throughout the water column, they were consistently detected at 10 m or below (Fig. 1, 2). In addition, there was a higher concentration of newly formed cysts and planozygotes during the sampling, in relation to duplets, which portrays life stages observed in the latter stages of the bloom.

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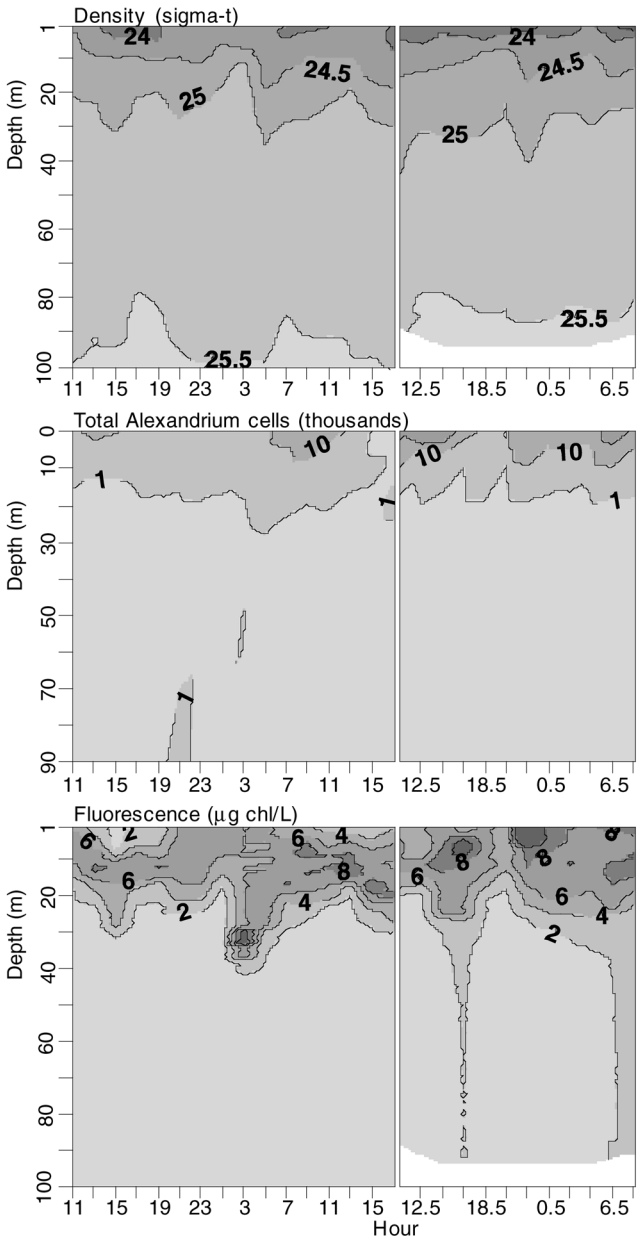

Figure 1 Contour plots of the temporal variation in the vertical distribution of water density, total abundance of all life stages of *A. fundyense* and fluorescence during July 12 and 13, 2001 (left panels) and July 16 and 17, 2001 (right panels).

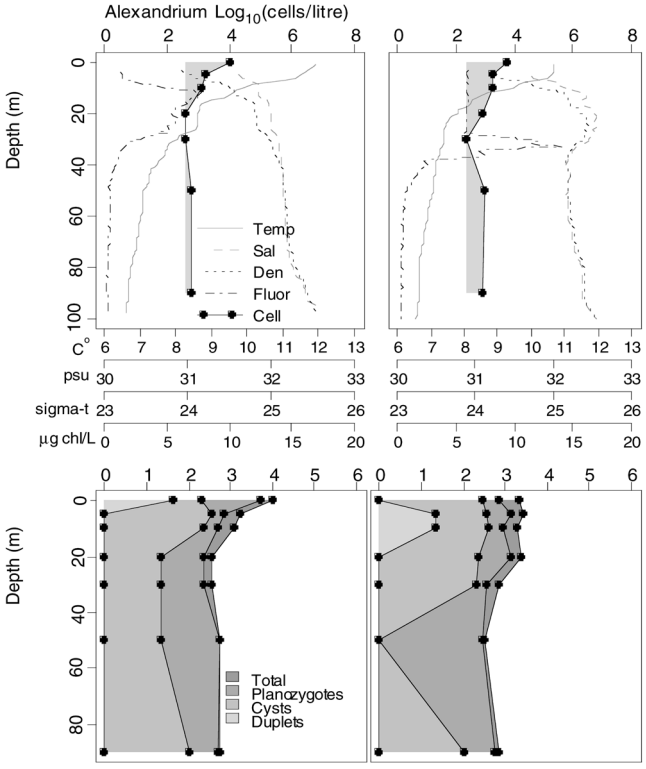

Figure 2 Vertical depth profiles (upper panels) of *A. fundyense* cells, salinity, water density, and fluorescence. *A. fundyense* profiles for all life stages and total are shown in lower panels. Plots contrast day (left panels; 1500 h) with night (right panels; 0300 h) on July 12 and 13, 2001.

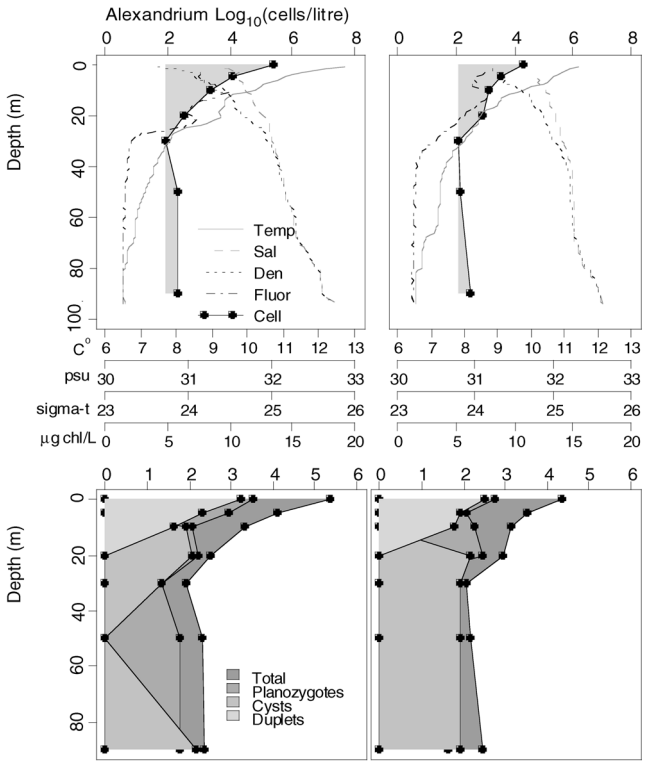

Figure 3 Similar to Fig. 2 but plots contrast day (left panels; 1430 h) with night (right panels; 0430 h) on July 16 and 17, 2001.

The water column in the Bay of Fundy during this study had a very shallow surface mixed layer and was weakly stratified with the base of the pycnocline at about 30 m (Fig. 1–3). Nitrate concentrations within the pycnocline or the very thin surface mixed layer were typically much less than 1 µM whereas nitrate concentrations below the pycnocline and at depth were significantly higher (5 µM). This indicates that *A. fundyense* cells were capable of existing at the lower nitrate concentrations (Fig. 4) and did not exhibit nocturnal descent. Although *A. fundyense* cells were observed throughout the water column, the highest concentrations consistently occurred above 10 m, either in the pycnocline

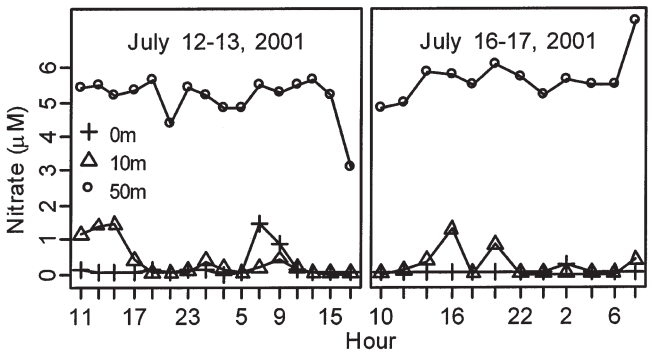

Figure 4 Nitrate concentrations at 3 depths.

or in the very thin surface mixed layer. Temperatures ranged from 10.5–12.3°C at the surface to 6.5–6.6°C at 100 m. Salinity values ranged from 31.5–32.6 psu throughout the depths.

In vivo fluorescence was not closely related to *A. fundyense* cell concentration (Fig. 1–3), but tended to coincide more closely with a *Pseudo-nitzschia delicatissima* "group" (includes *P. delicatissima* and *P. pseudodelicatissima*) bloom. This lack of linear relationship between fluorescence and *A. fundyense* densities confirms the necessity for identification and characterization of individual species when studying HABs with species-specific techniques.

Earlier studies suggest that the high concentrations of *A. fundyense* cells and cysts in seed beds in the offshore Bay of Fundy waters are responsible for elevated inshore shellfish toxicities (Martin and Richard, 1996). The ability of Bay of Fundy *A. fundyense* cells to remain in the upper layers may also be an adaptive strategy in their bloom behaviour for exploitation of coastal waters. The consistent restriction of cells to the upper layers of the water column observed through the 30- and 22-h samplings suggests the possibility for episodic wind-driven transport as a mode for advection to inshore shellfish areas along the coast.

Acknowledgements

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Phytoplankton Assemblages During Recurrent *Alexandrium minutum* Blooms in a Mediterranean Harbor

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Abstract

Alexandrium minutum causes recurrent and long-lasting blooms in Mediterranean coastal confined waters rich in nutrients. *A. minutum* concentrations were checked for six years (1996–2001) in Arenys de Mar, a Mediterranean harbor. During the blooms, a similar phytoplankton assemblage dominated by dinoflagellates was always observed. These dinoflagellates correspond to the life-forms Type I and II defined by Smayda and Reynolds in 2001, characterizing shallow, highly nutrient-enriched habitats.

Introduction

The determination of phytoplankton assemblages in communities containing HAB species is of major interest in order to understand which species are favored or compete for the same ecological niches or habitats (GEOHAB, 2001). Phytoplankton assemblages are not phylogenetic classifications of organisms, but are groupings of species according to their physiology, morphology or other features that respond similarly to recurrent patterns or factors (Estrada, 2000). Studies on phytoplankton assemblages in relationship to HABs in natural environments are scarce (Tilstone *et al.,* 1994), although there is a world-wide recognition of the relevance of this topic.

Alexandrium minutum is the most widespread toxic species in the Mediterranean basin. During the last decade, recurrent blooms were described in several harbors and shellfish farms (Honsell 1999; Vila *et al.,* 2001), including the Atlantic French coast (Probet 1999). The aim of the present paper is to analyze the phytoplankton assemblages during recurrent *A. minutum* blooms in the Arenys de Mar harbor (lat. 41°34′3″N; long. 2°33′3″E, NW Mediterranean Sea).

Materials and Methods

Arenys de Mar harbor was sampled during 6 annual cycles (1996–2001), once a week from May to October, and twice a month from November to April. Bucket phytoplankton samples (150-mL) taken from the surface were preserved with formaldehyde (1% final concentration) or Lugol's iodine solution and then settled and counted as described in Throndsen (1995). The phytoplankton community was analyzed during the *A. minutum* bloom development (pre-bloom: $<10^5$ cells · L^{-1}, bloom: $>10^5$ cells · L^{-1}, and post-bloom: <5,000 cells · L^{-1}).

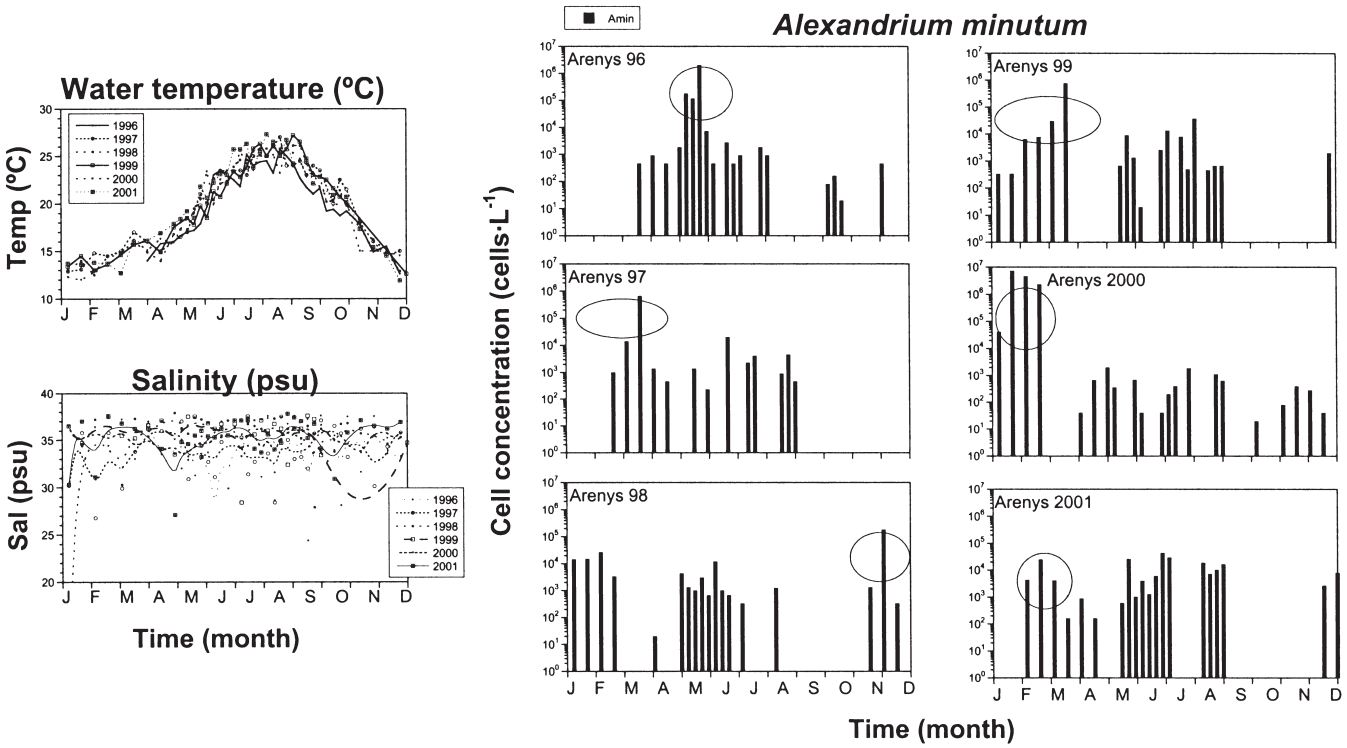

Figure 1 *Alexandrium minutum* cell concentration and water temperature in Arenys de Mar harbor (1996–2001). Circles indicate the blooms detailed on Fig. 2, together with other species in the phytoplankton assemblage.

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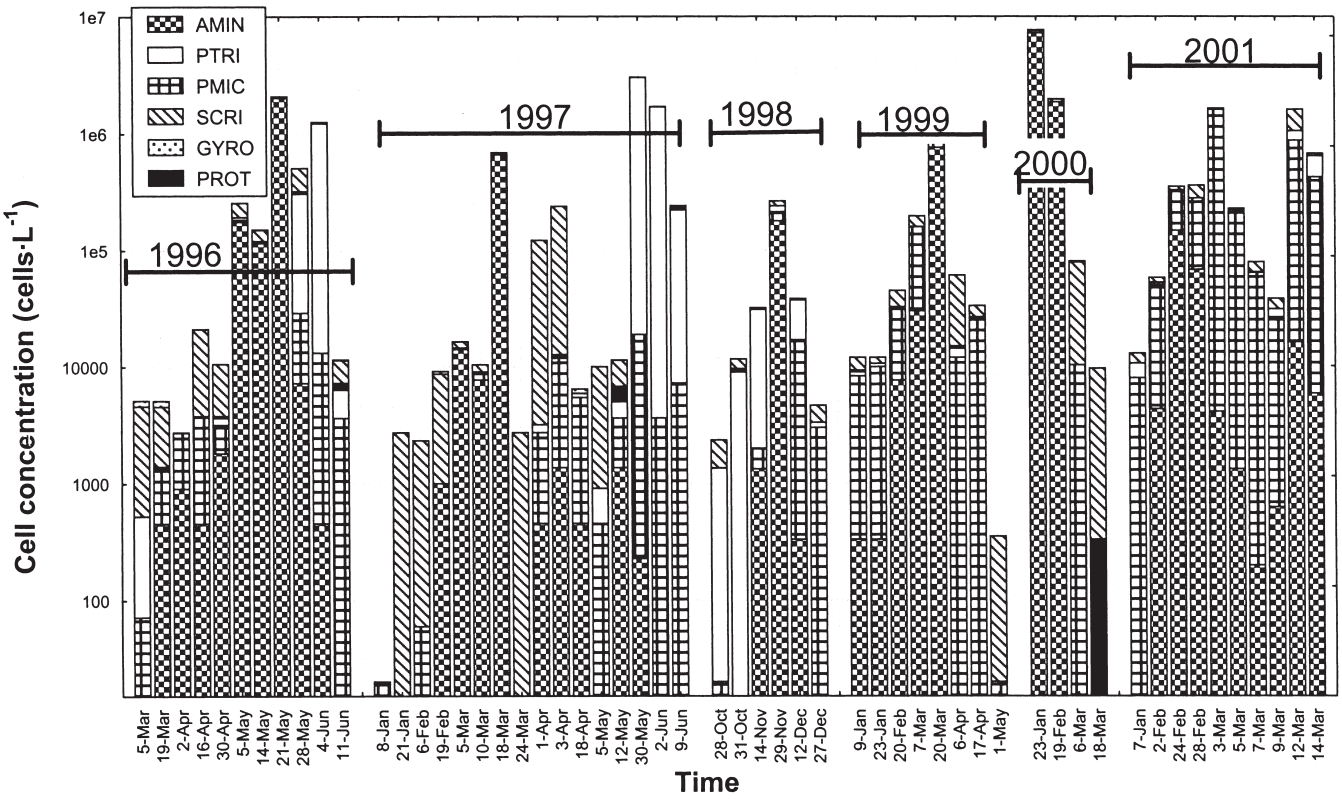

Figure 2 Cell concentration of the six dominant dinoflagellates during the *A. minutum* blooms indicated on Fig.1. The dominant co-occurring species were selected based on their maximum cell concentrations (over 10^4 cells · L^{-1} on at least one occasion). AMIN: *Alexandrium minutum,* PTRI: *Prorocentrum triestinum,* PMIC: *Prorocentrum micans,* SCRI: *Scrippsiella*-like species, GYRO: *Gyrodinium* cf. *spirale* and PROT: *Protoperidinium* sp.

Arenys de Mar harbor is an important fishing harbor with continental runoff and groundwater seepage inputs. This harbor is characterized by high inputs of inorganic nutrients. Mean values (± sd) of salinity (35.1 ± 2), chlorophyll *a* (5 μg · L^{-1} ± 3.6) and inorganic nutrients in surface waters of the harbor sampled monthly during two annual cycles (2000–2001, n = 22) were 0.88 μM ± 1 PO_4; 4 μM ± 1.6 NH_4; 102 μM ± 78 NO_3; 0.3 μM ± 0.1 NO_2; 25 μM ± 16 SIO_4.

Results

Alexandrium minutum is a species well adapted to Arenys de Mar harbor, where it blooms every year and water becomes reddish almost every year, coinciding with cell concentrations over 10^6 cells · L^{-1}. The bloom usually occurs in February–March (1997, 1999, 2000 and 2001 events) with surface water temperatures approximately about 14°C. However, it can occur at higher temperatures, as it happened in 1996 and 1998 events (Fig. 1). There is not a clear correlation with salinity, and blooms are recorded during wide salinity fluctuations. Blooms last up to 2 or 3 months.

The dominant accompanying species to such blooms are always dinoflagellates such as *Prorocentrum triestinum, P. micans* and *Scrippsiella*-like species—including also *Ensiculifera* sp. and *Pentapharsodinium* sp.—(over 10^6 cells · L^{-1}), and also smaller concentrations of *Protoperidinium* sp. and *Gyrodinium* cf. *spirale* (over 10^4 cells · L^{-1}). Other species such as *Dinophysis sacculus, Pseudo-nitzschia* spp., *Leptocylindrus danicus, Skeletonema costatum,* small *Chaetoceros* spp., *Eutreptiella* sp. and *Mesodinium* cf. *rubrum,* among others, are detected usually in lower concentrations (between 10^2-10^4 cells · L^{-1}).

In spite of the interannual variability, some trends can be observed in the 1996–1997 and 1999–2001 blooms (Fig. 2). During those years, in the pre-bloom period, the percentage of occurrence of *A. minutum, Scrippsiella* spp. and *P. micans* ranged from 10% to 57%, taking into account the dinoflagellate community. Meanwhile, *P. triestinum* never exceed 8%. In contrast, during the pre-bloom period of the autumn bloom in 1998, the dominant species was *P. triestinum* (91%). It was also observed that during the maximum concentrations of *A. minutum,* in general when they exceeded 10^5 cells · L^{-1}, blooms were almost monospecific (up to 96% of the microphytoplankton abundance was *A. minutum*). On the whole, *Scrippsiella* spp. and *P. micans* were the most represented taxa during pre- and post-blooms, except for the 1998 event. Instead, *P. triestinum* was often scarce during *A. minutum* events.

Discussion

Arenys de Mar harbor is a shallow, highly nutrient-enriched habitat that has a reduced water-mass exchange

with offshore waters. This habitat is characterized by recurrent *A. minutum* blooms that correspond to the life-form Type I defined by Smayda and Reynolds (2001). During the six-year period investigated, the phytoplankton assemblage associated with *A. minutum* blooms included life-form Type II, such as *P. triestinum, P. micans* and *Scrippsiella* spp., which characterize habitats where "nutrient levels are somewhat lower, but still high" (Smayda and Reynolds, 2001). The four dominant species from this phytoplankton assemblage would correspond to the red tide-forming group defined by Margalef *et al.* (1979). Those dinoflagellates bloom in unusual natural circumstances in which low turbulence is associated with high nutrient levels. The modification of the natural coastline and the subsequent increase in confined Mediterranean water areas, together with their high nutrient concentrations, favor environmental niches for dinoflagellate blooms as life-form Type I and II.

The dominance of one species is probably determined by slightly different environmental conditions that define the physical habitat, and also by interspecific competition, allelopathic and anti-predation defense mechanisms (Smayda, 1997). In this selective process, the best-adapted species will succeed. In this study, *A. minutum* bloomed preferably during winter-spring and dominated the microphytoplankton community after a pre-bloom period characterized by relatively high cell concentrations of *A. minutum, Scrippsiella* spp. and *P. micans.* However, in 1998 an *A. minutum* event occurred in autumn. In this occasion, the pre-bloom community was dominated by *P. triestinum,* and *A. minutum* did not reach maximum cell concentrations (10^5 cells $\cdot$ L^{-1} detected only once). However, since that bloom occurred in a completely different season (late autumn), the effect of the seasonality on the community structure could not be ruled out.

More field studies following *A. minutum* assemblages in other localities and seasons are necessary in order to determine if the association detected here is or is not a general rule. Anyway, we have to keep in mind that the precise community composition is, in practice, impossible to predict and that the dominance of the best-adapted species is considered to be only a probability (Smayda and Reynolds, 2003). In the future, the availability of long time series of the phytoplankton assemblage including harmful species will provide valuable information to determine if a given phytoplankton community favors harmful algal blooms.

Acknowledgements

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Patterns of Short-Term and Long-Term Accumulation of Phycotoxins in Zooplankton Feeding on *Alexandrium fundyense*

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Abstract

Zooplankton grazers can accumulate phycotoxins when feeding on harmful algae, potentially exposing higher trophic levels of food webs to inimical or life-threatening levels of bioactive compounds. Paralytic shellfish poisoning (PSP) toxins produced by *Alexandrium fundyense* have been detected in wild zooplankton populations in the Gulf of Maine. To assess the potential for vectoral intoxication of marine food webs by zooplankton, laboratory experiments were performed with copepods (the dominant mesozooplankton) common to the coastal Gulf of Maine, feeding on diets containing toxic *A. fundyense,* over short (days) and long (weeks) time periods, to determine rates of feeding (toxin ingestion) and toxin accumulation (measured by HPLC-FD). In all experiments, retention of toxins was remarkably inefficient (<5% of toxin ingested). Toxin accumulation in copepod tissues was not initially rapid, and was also not steady, characterized by lags and subsequent increases in toxin body burden, generally corresponding to diel feeding periodicity. Potentially dangerous levels of toxin were accumulated by zooplankton feeding on mixed diets, but loss of toxin was often rapid in active zooplankton. Laboratory experiments and field-collected zooplankton showed that although maximal body burdens of toxin may accumulate in copepods within days of exposure to *A. fundyense,* these burdens can vary by an order of magnitude over daily cycles. Copepods are not efficient vectors of phycotoxins, but they may remove toxic *A. fundyense* from the water, accumulate modest body burdens of toxin, and depurate quickly.

Introduction

Mesozooplankton such as copepods usually dominate net zooplankton biomass in the Gulf of Maine, and are the dominant trophic link between microplankton and planktivorous fishes and marine mammals. Paralytic shellfish poisoning (PSP) toxins originating from the dinoflagellate *Alexandrium fundyense* have been detected in wild populations of zooplankton from the Gulf of Maine, and fish and marine mammal mortalities have been attributed to consumption of intoxicated zooplankton acting as vectors of toxin (White 1981; Geraci *et al.,* 1989). Several studies have shown that toxin accumulation in copepods is inefficient (Teegarden and Cembella 1996; Guisande *et al.,* 2002; Teegarden *et al.,* 2003), but little is known about the dynamics of toxin accumulation in zooplankton over the time scales of *A. fundyense* blooms, the relationship between zooplankton feeding and toxin accumulation, and the magnitude and duration of the threat of vectoral transfer of toxins via zooplankton. In this study laboratory experiments were performed to determine patterns of PSP toxin accumulation over days to weeks (the time scales of *A. fundyense* blooms in the coastal Gulf of Maine), and the relationship of feeding patterns and toxin accumulation patterns. Most of these experiments used mixed phytoplankton diets, since monospecific blooms of *A. fundyense* are rare in natural conditions. Results were compared with zooplankton samples collected from the field during a bloom of *A. fundyense* to assess whether laboratory results reflect actual accumulation levels in the field.

Materials and Methods

Phytoplankton were cultured at 15°C, 14:10 light:dark cycle. Strains used were *Alexandrium fundyense* strains GTCA 28 (toxicity 15–22 pgSTXeq cell^{-1}) and BC1 (toxicity (55–85 pgSTXeq cell^{-1}), *Heterocapsa triquetra* HT984, *Rhodomonas salina* CCMP1312, and *Thalassiosira weissflogii* B9TW. The copepods *Acartia hudsonica, Centropages hamatus,* and *Eurytemora herdmani* were maintained in culture as detailed in Teegarden (1999) to ensure toxin-free initial

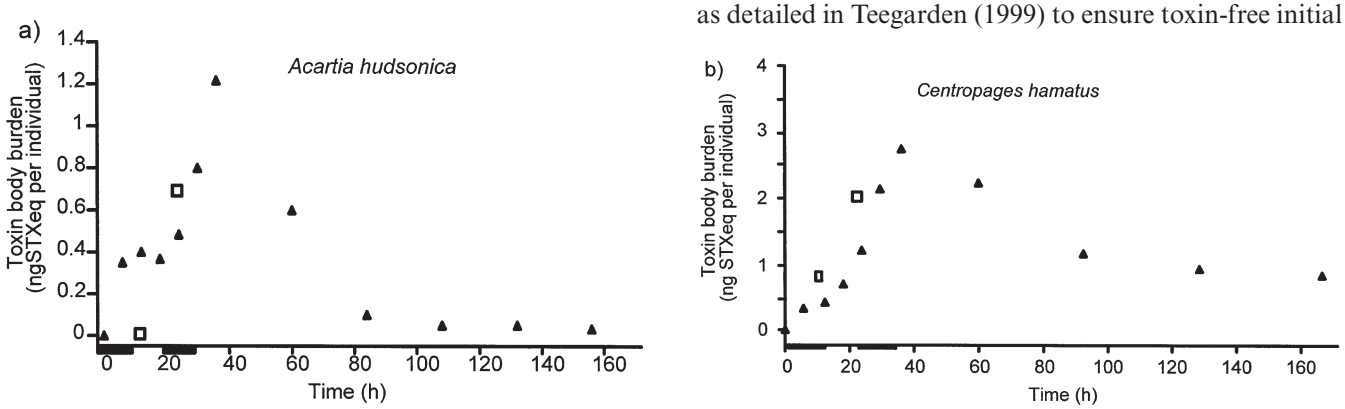

Figure 1 Toxin body burden of copepods **a** *Acartia hudsonica* and **b** *Centropages hamatus* over 36-hour feeding on monoculture *A. fundyense* and week-long detoxification with a diet of *H. triquetra.*

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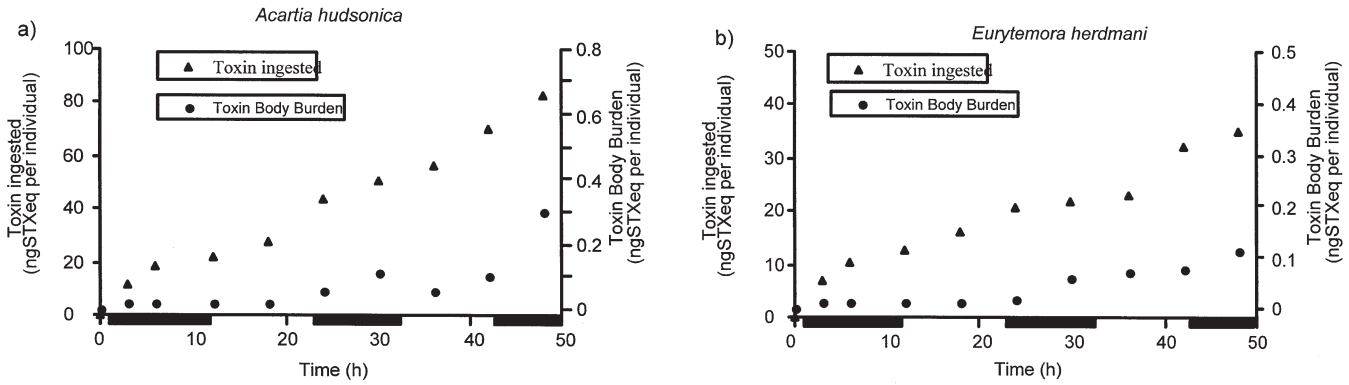

Figure 2 Toxin ingested (Y1 axis) and toxin accumulated in body tissues (Y2 axis) by **a**, *Acartia hudsonica* and **b**, *Eurytemora herdmani* over time in 48-hour feeding experiments.

samples. Healthy adult females were used for experiments. All experiments (except long-term accumulation experiments) used a plankton wheel rotating at 1 rpm to ensure even distribution of food items, 14h:10h light:dark cycles, and triplicate (for each sampling period) 500–1000 mL grazing containers with 10–20 copepods per container as well as control containers with no grazers. At each sampling, copepods from the three containers were isolated into vials (one vial per container, 10–20 copepods per vial) and frozen for later extraction. Samples of the algal mixtures were preserved with Lugol's solution, and algal concentrations were counted with a microscope and Sedgwick-Rafter cell or various settling chambers. Integrated ingestion and clearance rates were calculated with the Frost (1972) equations. Samples of algae and copepods were extracted in 0.1 N acetic acid and 70% methanol, respectively, and analyzed for toxin content and composition (carbamate, sulfocarbamoyl, decarbamoyl derivatives) by high performance liquid chromatography with fluorescence detection (HPLC-FD), as detailed in Teegarden *et al.* (2003).

Monoculture Accumulation/Detoxification Experiments
Copepods were fed a monoculture diet of *A. fundyense* GTCA 28 for 36 hours (300 µg C L^{-1}), then transferred to *H. triquetra* in 20-L culture containers for 7 days. Samples were taken every 6 hours during feeding on *A. fundyense,* and daily during the detoxification stage. During toxification stage, at 12 and 24 hours two sets of samples were collected, one for immediate processing, while the other samples of copepods were allowed to incubate in *H. triquetra* for 3 hours, to account for loss of gut contents.

Mixed Diet Ingestion/Accumulation Experiments Copepods were fed a mixed diet of *A. fundyense* BC1 and *H. triquetra* (200 µg C L^{-1} each) for 48 hours, with sampling at 0, 3, and 6 hours, and every 6 hours thereafter.

Long-Term Accumulation Experiments Copepods were fed a mixture of *A. fundyense* GTCA 28, *H. triquetra, T. weissflogii,* and *R. salina* (100 µg C L^{-1} each) in 20-L culture containers, with algae counted and replenished daily to maintain concentrations, for 7 days, with daily sampling. Copepods were then transferred to fresh solutions of the above algal mixture without *A. fundyense,* and sampled every 12 hours for three days, then daily for four days.

Results and Discussion

Monoculture Accumulation/Detoxification Experiments
Copepods accumulated PSP toxins in a stepped pattern over 36 hours, with increases in toxin body burden especially during dark periods, followed by lags or plateaus during light periods (Fig. 1a,b). *Acartia hudsonica* reached a maximum body burden of about 1.2 ngSTXeq per individual after 36 hours, while *Centropages hamatus* reached a body burden of 2.7 ngSTXeq per individual, owing to larger body size and probable higher ingestion rates. Open squares in Figs. 1a and b indicate average toxicity of individuals from groups that were removed at the time indicated, and allowed to clear their guts for 3 hours. In most cases, toxicity of individuals was actually higher after incubation in alternate food, a result of transformation of less potent n-sulfocarbamoyl toxin derivatives in tissues to carbamate derivatives. After 48 hours and removal to alternate food, *A. hudsonica* lost toxin body burdens over two days, equal to the time of accumulation, while in *C. hamatus,* loss of toxins was initially rapid, but after reaching just under half of the maximum attained, slowed dramatically, as toxins remained in tissues throughout the sampling period.

Mixed Diet Ingestion/Accumulation Experiments To ascertain whether the stepped pattern observed above was a result of feeding periodicity, toxin ingestion and toxin accumulation in tissues were simultaneously measured. Fig. 2a and b show the stepped pattern of toxin accumulation observed in previous experiments, and feeding patterns typical of calanoid copepods with higher ingestion during dark periods and lower ingestion during light periods (indicated by increases in toxin ingested, followed by steady or unchanging levels of toxin ingested). Despite ingestion of moderate to high quantities of toxin, copepods retained only a small fraction of toxins (N.B. scale of body burden Y2 axis compared with scale of ingestion Y1 axis), less than 1% of ingested toxin. During periods when copepod ingestion rates were low, toxin body burdens of copepods

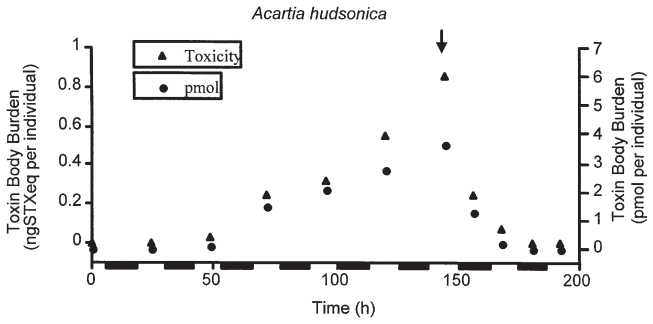

Figure 3 Accumulation of toxin in *Acartia hudsonica* in long-term accumulation experiments. Dark bars indicate dark periods.

could actually decline, indicating rapid loss of toxins even in feeding copepods.

Copepods did not display significant selective feeding, ingesting similar quantities of both *A. fundyense* and *H. triquetra.* Compared with copepods fed monoculture *A. fundyense* GTCA 28 diets for 36 hours, copepods feeding on the mixed diet of *A. fundyense* BC1 and *H. triquetra* for 48 hours attained lower toxin body burdens, despite the fact that the BC1 clone of *A. fundyense* was more toxic. This was likely a result of lower total ingestion of toxic cells in mixed diets compared to monoculture diets, as numerous experiments with BC1 and GTCA 28 have shown no difference in toxin retention efficiency (Teegarden *et al.,* 2003).

Long-Term Accumulation/Detoxification Experiments When fed mixtures of phytoplankton containing only 25% *Alexandrium fundyense* (μg C L^{-1}), *Acartia hudsonica* required a full week to attain the toxin body burdens attained within two days in more concentrated monoculture and two-species diets of previous experiments (Fig. 3). After several days, toxicity of copepod tissues (ngSTXeq) increased faster than toxin concentration (pmol), as retained n-sulfocarbamoyl C-toxins were converted to gonyautoxin (GTX) carbamate derivatives. After removal to non-toxic phytoplankton mixtures (arrow in Fig. 3), *A. hudsonica* lost toxins in tissues within two days.

Field-Collected Zooplankton Table 1 shows toxin values of copepod tissues in μgSTXeq per g wet weight of bulk samples dominated by *A. hudsonica,* as well as toxin content per individual from individually picked adult female *A. hudsonica* during a bloom of *A. fundyense* in Cundy's Harbor, Casco Bay, Gulf of Maine. Total toxin accumulations are similar to maxima attained in laboratory experiments with either monoculture or mixed diet experiments. During this bloom, *A. fundyense* was a minor portion of the available food, never more than 25% of available carbon. Both within and between sampling dates, toxicity of *A. hudsonica* tissues varied by an order of magnitude, indicating temporal and spatial patchiness of intoxicated zooplankton, and suggesting dynamic accumulation and loss of toxin as found in laboratory experiments.

Table 1 Toxin content of field-collected *Acartia hudsonica* during *A. fundyense* bloom.

Date	ngSTXeq per individual	pgSTXeq per g wet weight
5/18/98	0.777	1.807
5/18/98	0.095	0.221
5/25/98	0.616	1.432
5/25/98	0.074	0.173
6/2/98	0.216	0.502
6/2/98	0.102	0.238
6/2/98	1.208	2.810
6/8/98	0.796	1.851

Conclusion

Kinetics of PSP toxin accumulation and toxin body burdens in marine copepods reflect feeding patterns, and vary over diel cycles. Retention of PSP toxins by copepods is poor, as only a minor fraction of toxins calculated as ingested ever appear in body burden. Relatively high body burdens are attained rapidly with monoculture diets. In mixed diets more representative of natural conditions, a week or more may be needed before body burdens are attained that are comparable to typical field-collected copepod levels. Though variable, field populations do not attain body burdens that are significantly different from laboratory studies, despite weeks of exposure to toxic *Alexandrium fundyense.* Toxin loss in copepods may be rapid once toxic food is depleted, or even when feeding rates slow, though this response may depend on copepod species. Assessing the threat posed by accumulation of toxins in copepods and possible transfer to other parts of marine food webs, we conclude that although potentially inimical levels of toxin are retained in copepod tissues, 1) toxin accumulation is temporally and spatially variable, and takes place over time scales similar to those of bloom duration (weeks), 2) loss of toxin from tissues is probably rapid in active copepods, and 3) poor retention of toxins suggests that copepods are not efficient vectors of toxins.

Acknowledgements

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The Resting Cyst of *Alexandrium catenella,* a Dinoflagellate Responsible for Harmful Algal Blooms in Thau Lagoon (Western French Mediterranean Coast)

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Abstract

For the first time, the sexual resting cyst of the dinoflagellate *Alexandrium catenella* was isolated and described from surface sediments of Thau Lagoon (French Mediterranean coast). During the two periods of January–May 2000 and 2002, *A. catenella* cysts were sampled and quantified from sediments in several locations of the lagoon. This preliminary study has revealed that all the sampling stations contained *A. catenella* cysts. A small embayment (Angle Creek) showed the highest cyst densities and seems to be a favourable area for accumulation of these dormant stages. Different sampling and quantification methods were compared to improve the estimation of cyst abundance.

Introduction

In Thau Lagoon, the first outbreak of PSP caused by *Alexandrium* sp. was reported in 1998 (Masselin *et al.,* 2000). The associated measured toxicity was up to 852 µg STX eq 100 g^{-1} of mussel meat. *A. catenella* has been shown to be responsible for the recurrent blooms since this first toxic event (Barré, 2001; Lilly *et al.,* 2002). In Mediterranean coastal waters, species of the genus *Alexandrium* occur frequently in small embayments or shallow waters (Garcés *et al.,* 1999). The life cycle of *Alexandrium* species is characterised by a sexual phase, producing hypnozygotes which sink and produce benthic resting cysts. The distribution of cysts in sediments is a useful biological parameter for the monitoring surveys of toxic dinoflagellate species (Anderson and Wall, 1978). Resting cysts play an important role not only in dinoflagellate dispersal, initiation and termination of blooms, but also in survival under unfavourable conditions (Dale, 1983). The fate and distribution of deposited cysts are important variables necessary to understand the ecology and bloom dynamics of toxic species (Hallegraeff, 1993). The objectives of the present study were (1) to isolate and to determine systematically *Alexandrium* sp. cysts from Thau, (2) to compare different sediment sampling and cyst quantification methods and (3) to establish a first distribution of *Alexandrium* cysts in some sensitive areas in Thau Lagoon, especially in Angle Creek, where major cell concentrations are regularly recorded.

Material and Methods

Sample Area Thau Lagoon is a large Mediterranean water mass, 14 km long and 5 km wide. Its mean depth is 4.5 m, with a maximum of 10 m. Three channels link the lagoon to the sea; 80% of exchanges are through the Sète channels (Fig. 1). Sampling of sediment was conducted from January to May in 2000 and 2002. Three locations were selected: (A) Angle Creek, a small embayment to the north where the highest cell concentration of toxic *A. catenella* was recorded (350,000 cells · L^{-1} in 1998), and two locations out of Angle Creek: (B) Bouzigues, an important shellfish production area, and (S) a station near Sète. For this preliminary study, five stations were sampled: A_1, A_2, A_3 (Angle Creek), B (Bouzigues) and S (Sète).

Sediment Sampling, Characterisation and Preparation To obtain naturally occurring cysts, surface sediment was collected with an Eckman grab sampler (Yamaguchi *et al.,* 1995). The top 3 cm of sediment samples were placed in plas-

Stn	Lat. N	Long. E	Depth
A_1	43°27′364	003°40′509	0.9 m
A_2	43°27′081	003°40′402	2.2 m
A_3	43°26′920	003°40′344	3.3 m
B	43°26′261	003°39′508	8.4 m
S	43°25′460	003°39′359	7.5 m

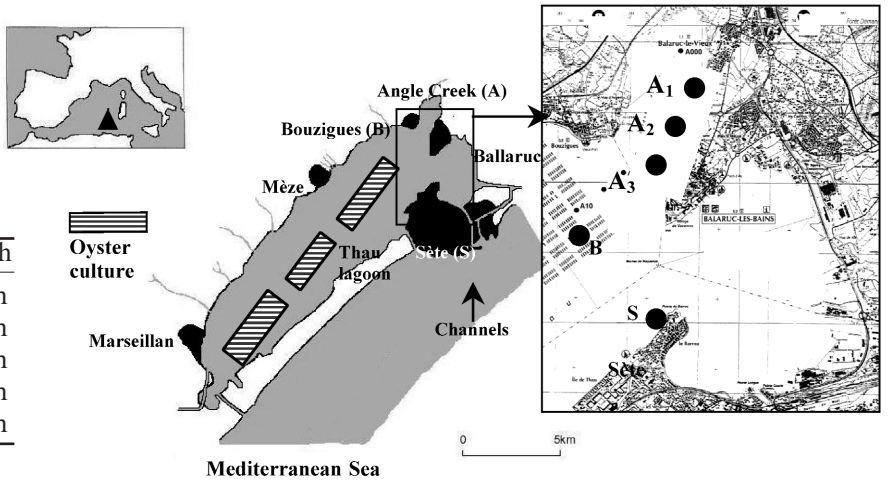

Figure 1 Map of Thau Lagoon and areas where samples were taken. See also geographic coordinates and depths of sampled stations.

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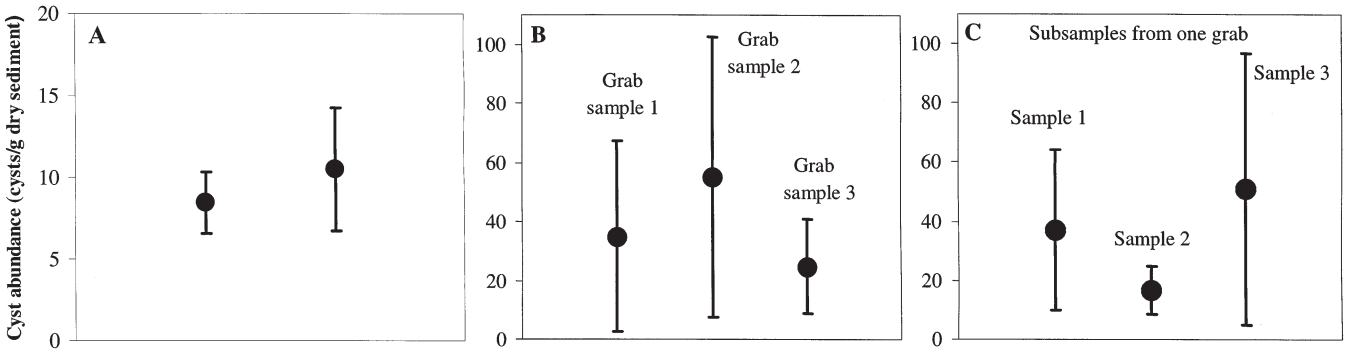

Figure 2 Variation of cyst abundance as a function of sampling method (**A**), number of grabs taken (**B**), or subsamples taken from a grab (**C**).

tic containers and stored in the dark at 4°C. To characterise the sediment, one subsample was oven-dried at 60°C to calculate the water content. The second subsample was lyophilised, weighed and sieved (63-µm mesh). The size fraction <63 µm was reweighed to determine the fine fraction. Two other subsamples were used for cyst counting, using the most probable number (MPN) and density-gradient methods (Erard-Le Denn and Boulay, 1995). After homogenisation, 1-g (wet weight) aliquots of the sediment subsample were suspended in filtered (0.2 µm) seawater, sonicated for 2 minutes to dislodge detritus particles and sieved through plankton nets to obtain the size fraction between 20 and 140 µm.

Germination-Dilution Technique According to the MPN method (Imai *et al.,* 1984), the sediment fraction passing the sieve (140 µm) was suspended in 10 mL filtered seawater. One mL of this suspension was added to five replicate tubes containing 9 mL f/2 medium (Guillard and Ryther, 1962). After three 10th serial dilutions with the same medium, these tubes were incubated at 20°C under 100 µE $\cdot m^{-2} \cdot s^{-1}$ and 12:12 h light : dark cycle. After five days, vegetative cells of *Alexandrium* sp. were examined under the inverted microscope (fifteen settling chambers). In this experiment, any tube with *Alexandrium* sp. motile cells was scored as positive.

Density-Gradient and Centrifugation Technique To separate the cysts from detritus, the density differences between cysts of *Alexandrium* sp. and that of the gradient medium Ludox CLX (d = 1.37 g cm^{-3}) (Erard-Le Denn and Boulay, 1995) were used. The band containing cysts was collected. This fraction was sieved through a 20 µm net and placed in a 5 mL tube containing distilled water and sonicated for 2 minutes. The resultant solution was used to enumerate cysts by the inverted microscope (1 to 3 settling chambers) using the Utermohl (1958) method.

Light Microscope Observations To identify systematically isolated cysts from Thau Lagoon, the morphology of germling cells was studied by light microscopy. Plate tabulation (Balech, 1995) was studied after dissecting a theca using 5% sodium hypochlorite. The length and width of at least 30 germling cells were determined.

Comparison of Sampling Methods We compared samples from an Eckman grab sampler and a gravity core sampler. Sediment samples derived from these methods were taken in A_2. We also compared the variability in cyst abundance within samples from three different grab samplers, and within three samples collected from the sediment of one grab. Each value represents at least three counts corresponding to three replicates.

Results and Discussion

Comparison of Sampling and Quantification Methods

Using the density-gradient method, results showed that the measured abundance of cysts in A_2 were not significantly different in the sediments collected either with an Eckman grab or core sampler (Wilcoxon-Mann-Whitney test, $P >$ 5%) (Fig. 2A). However, core sampling allows information to be obtained about the vertical profile of cyst accumulation. There was no significant difference between cyst abundance measured in the three grab samples (Fig. 2B). Similarly, cyst abundance determined from three subsamples from the sediment of one grab sampler was not significantly different (Fig. 2C) (Kruskal-Wallis test, $P >$ 5%). However, these results underlined the variability (see SD) in cyst distribution within one sediment sample, which implies the need for increased numbers of samples to improve quantification of cyst abundance in a defined area. Our results showed that MPN values were greater than those obtained by enumeration using the density-gradient method, but spatial distribution of cysts determined by the two methods of counting was nearly the same (Fig. 3). Erard-Le Denn and Boulay (1995) suggested that the dilution method could be applied to obtain an indirect count of viable cysts for rapid screening of an area. In conclusion, the two cited methods bring complementary information.

Cyst Features The resting cysts are cylindrical with rounded ends. The clear cyst wall is covered with mucilage, while the cell contains granular material, an orange-red accumulation body and numerous lipid globules (Fig. 4).

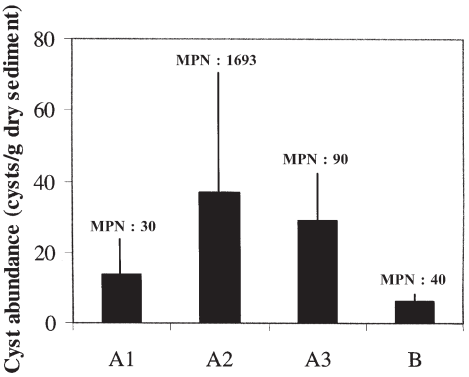

Figure 3 Abundance of *A. catenella* cysts in the stations sampled in Thau Lagoon. Abundance was calculated from counting with the density-gradient (histograms + error bars) and the MPN (MPN : value) methods.

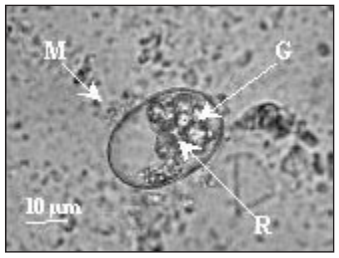

Figure 4 Photographic image of *A. catenella* cyst from Thau. (M) remaining mucilage, (G) granular material and (R) orange-red accumulation body.

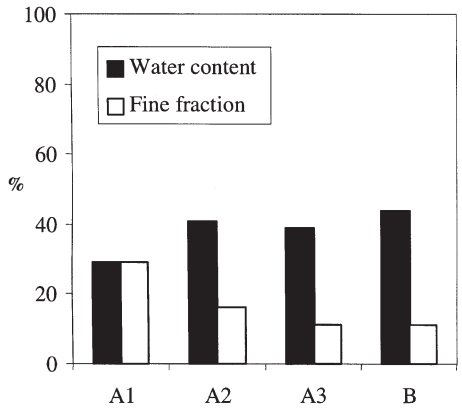

Figure 5 Water content and fine fraction percentages in the sediment sampled in different locations of Thau.

Central body was 41.86 ± 5.32 µm long and 30.4 ± 4.52 µm wide. Cysts of *A. catenella* and *A.tamarense* are indistinguishable in appearance and size (Fukuyo, 1985). The examination of the vegetative cells from germling cells was therefore necessary to determine the taxonomic affinity of the isolated cysts. Germling vegetative cells were round and covered by a cellulosic theca. Mean length and mean width were 26.15 ± 3.2 µm and 30.58 ± 4.28 µm, respectively. Plate formula refers to both *A. catenella* and *A.tamarense* (Fukuyo, 1985). The vegetative cells showed the absence of a ventral pore between the first and the fourth apicals and the ability to form chains of two to eight cells, suggesting that the isolated cysts from Thau Lagoon correspond to *A. catenella*.

Spatial Distribution and Ecological Implications On the basis of cyst densities obtained using the density-gradient method, all the sampling stations contained *A. catenella* cysts. Angle Creek seems to be favourable to deposition and in accumulation of cysts (A_2>A_3>A_1>B) (Fig. 3). MPN counts showed that all the sampled areas including the station S (70 cysts/g^{-1} dry sediment) had *A. catenella* present. The number of cysts was not related to the fine fraction or water content of the sediment in stations $A_{1,2,3}$ and B ($r^2 < 0.03$, $P = 0.05$). However, these results have to be taken with caution because the sampled areas have similar sediment characteristics (Fig. 5) (Erard-Le Denn and Boulay 1995). Further measurements have to be performed throughout Thau Lagoon and must consider hydrographic processes and sediment dynamics.

The presence of *A. catenella* cysts in the sediment of Thau Lagoon may provide a concentrated inoculum for subsequent development of toxic blooms. The maximum observed cyst abundance was 130 cysts per g^{-1} of dry sediment and was found in A_2. Both MPN and density-gradient methods revealed that Angle Creek and particularly A_2 had the highest cyst density. This finding corresponded to the *in situ* distribution of vegetative cells during the recurrent blooms of *A. catenella* in Thau Lagoon (Vaquer, pers. comm). The depth of Angle Inlet is <4 m; this would allow the sedimentation of the new formed hypnozygotes and their subsequent excystment. It seems likely that sediment resuspension locally induces the germination of resting cells and the initiation of a potentially toxic bloom event. Sediment collected from stations B and S contained *A. catenella* cysts. This suggests the extension of distribution of cysts which can be produced locally by motile cells or transported from other previously infected areas. An exhaustive study of cyst abundance and distribution in Thau Lagoon would have to be performed to know the accumulation and dispersion of cyst seedbeds of *A. catenella*.

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Correlation of *Karenia brevis* Presence in the Eastern Gulf of Mexico with Rainfall and Riverine Flow

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Abstract

Counts of *Karenia brevis* from the eastern Gulf of Mexico between 1953 and 1998, binned by months and converted to presence/absence and duration, were analyzed for cross-correlation with rainfall and riverine flow. The region was divided into three sub-areas for analysis to accommodate geographic variation in potential forcing factors. The duration of the presence of *K. brevis* in northern Florida waters was not significantly related to either rainfall or flows. The central Florida region recorded significant correlations of *K. brevis* durations with a number of central Florida rainfall stations and with flows of most central and southern rivers examined. Lags were short (less than two months), if any, indicating relatively direct linkages. Relationships of *K. brevis* in southern Florida waters to rainfall, while similar to the central region, were significantly correlated for only a few south Florida rainfall stations and primarily with flows from the Peace River in south central Florida. Cross-correlations of *K. brevis* duration with departures of flow or rainfall from seasonal means were generally not significant.

Introduction

In the Gulf of Mexico, the dominant organism responsible for red tides is *Karenia brevis* and its presence has been inferred as early as 1844 (Feinstein, 1956). A number of mechanisms for the perceived recent increases in blooms have been proposed, including an increase in nutrient loadings to coastal waters by both anthropogenic activities and long-term climatic cycles. This work examined presence of *K. brevis* in the eastern Gulf of Mexico for relationships to rainfall and riverine flows as surrogates for nutrient or trace requirement loadings. The implicit null hypothesis was that *K. brevis* presence and duration was uncorrelated with these dependent variables.

Materials and Methods

A 46-year record of counts of *K. brevis* (1953–1998), assembled by the Florida Marine Research Institute from multiple sources (FWC–FMRI, 2001), were segregated by region (Fig. 1A) and reduced to presence/absence by month to avoid biases due to varying sampling effort and uncertainties as to reporting units of cell concentrations.

Data were available from 75% of the months for Region II, but for only 10% and 52% of the months for Region I and Region III, respectively. Missing data were conservatively replaced with "absence." A three-month duration parameter was computed by region as the number of months of the following 3 months in which *K. brevis* was present. Duration parameters were similarly computed for 6, 9, 12, 18, and 24 month accumulation periods. Flows were obtained from 22 US Geological Survey stations (Fig. 1B, Table 1). Monthly rainfalls were obtained from 41 National Weather Service cooperating stations from Pensacola to Key West (Fig. 1C), and within the contributing watershed. Cumulative flows and rainfalls over a variety of periods (3, 6, 12, and 24 months), as well as anomalies (departure from monthly means), were also investigated. *K. brevis* duration periods and hydrologic accumulation periods were selected to represent a range between relatively immediate to improbably long linkages between *K. brevis*

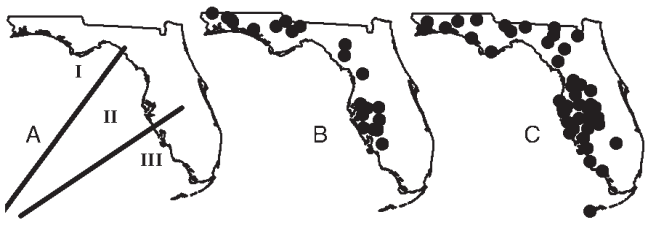

Figure 1 **A** Regions of the eastern Gulf of Mexico. **B** Locations of riverine flow stations. **C** Locations of rainfall stations.

Table 1 Riverine flow stations

#	Location	Region
21	Escambia River near Century	I
20	Blackwater River near Baker	I
19	Yellow River near Milligan	I
18	Shoal River near Crestview	I
17	Choctawhatchee River near Bruce	I
16	Chipola River near Altha	I
15	Appalachicola River at Chattahoochee	I
14	Ochlockonee River near Havana	I
13	Ochlockonee River near Bloxham	I
12	Suwannee River at Branford	II
11	Suwannee River Wilcox	II
10	Withlacoochee River near Holder	II
9	Hillsborough River near Tampa	II
8	Alafia River at Lithia	II
7	Little Manatee River near Wimauma	II
6	Myakka River near Sarasota	III
5	Peace River at Bartow	III
4	Peace River at Zolfo Springs	III
3	Peace River at Arcadia	III
2	Horse Creek near Arcadia	III
1	Joshua Creek at Nocatee	III
0	Caloosahatchee River, S-79	III

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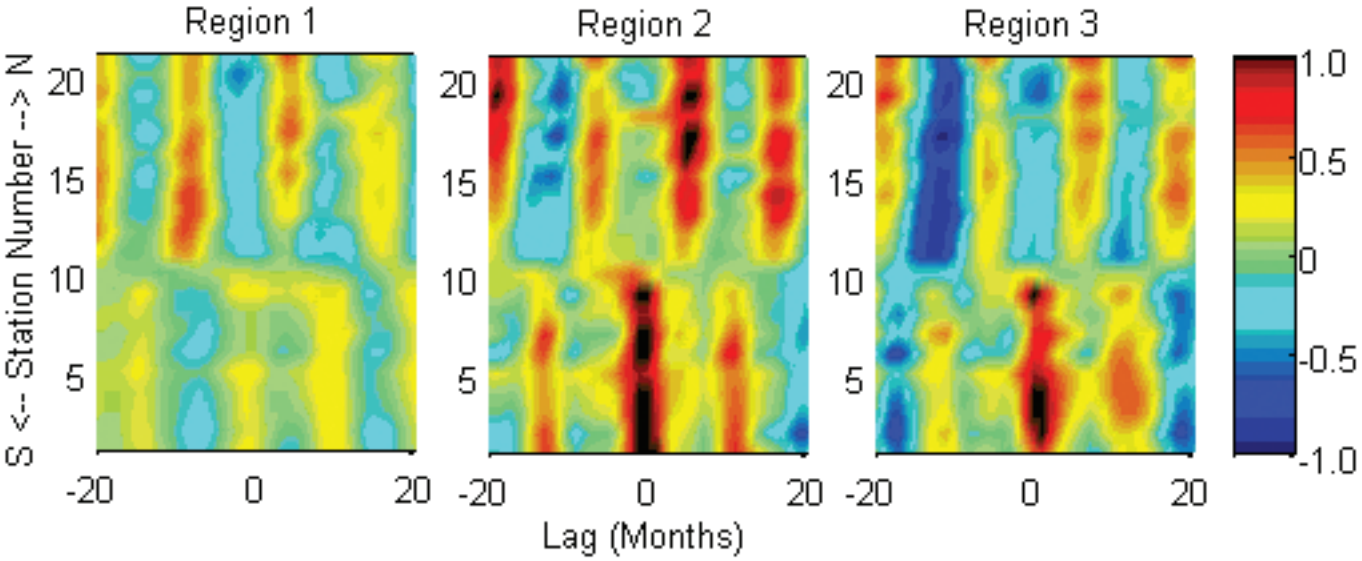

Figure 2 Geographically compiled, normalized cross-correlations of the three-month *K. brevis* duration parameter against riverine flow, by region. Values ≥1.0 (black) are significant at $P = 0.05$.

and hydrological factors. Flow data from the Caloosahatchee River were only available since the 1960s, so analyses were repeated on the shorter time period.

Cross-correlation analyses evaluated *K. brevis* duration by region in relation to flow and rainfall variables for both positive and negative lag periods. When interpreting the lag times of cross-correlation analyses for information on initiation of blooms, the duration parameters are phase shifted by the number of months of accumulation less one, *i.e.,* given a single month in which *K. brevis* occurred, a 24-month duration parameter would have a value of 1 at 23 months prior to the actual occurrence, and would remain at 1 until the month of occurrence. Significance levels of cross-correlation were based on the more restrictive N* (Chelton, 1983) to correct for serial correlation within the various data sets. Correlation results of regional *K. brevis* durations with individual rainfall or flow stations were normalized to the value required for $P = 0.05$ and compiled geographically, from north to south, for illustration.

Results and Discussion

Hydrology of the west coast of Florida exhibited two dominant modes of seasonal distributions. Central and south Florida rainfall sites experienced maxima during the summer months. Flow maxima for the Withlacoochee River and rivers to the south occurred in August–October. Rainfall in northern Florida exhibited a bi-modal distribution with summer and winter wet periods. Flow maxima for northern rivers were received in March–April. The mean annual flows were generally higher for the northern than the central and southern rivers.

Durations of *K. brevis* in Region I over a three-month period were not significantly correlated with riverine flows. In Region II, however, three-month duration as a function of flow was significant at –1 to 0 month lags for a number of central and southern rivers and at 5–6 months lags for some northern rivers (Fig. 2). *K. brevis* appearing to 'lead' flow (lag of –1) was the product of the phase shift of the duration parameter (in which duration for the coming three months was compared to flow during the present month) and indicated that linkages with flow, if causal, were relatively immediate. While the lags at which northern river flows were significant may reflect the relative timing of flows from north to south, there were important interannual differences such that calculated significance values were not symmetrical about zero lag. Region III exhibited significant relationships with a few southern rivers, and those at zero to one month lags, or slightly delayed from blooms in Region II. Longer durations of *K. brevis,* cumulative flows, or flow departures did not produce improved significance. Neither the largest discharge nor the most highly managed river produced significant correlations with *K. brevis* durations.

The three-month *K. brevis* duration in Region II was strongly linked to rainfall in the central portion of the state, from approximately Tarpon Springs to Punta Gorda (rainfall stations 6–26; Fig. 3). Significant lags ranged from –1 to +2 months. Both *K. brevis* duration and monthly rainfall have a strong seasonal signal (apparent at 12 months), but correlations are only significant at short lags, indicating important interannual differences in both parameters. Examination of longer duration parameters, longer accumulation periods of rainfall, or rainfall anomalies did not provide any increases in significance. Region III *K. brevis* occurrences exhibit relationships with rainfall from similar geographic regions as does Region II, but few are significant. Region III occurrences have even less relationships with northern rainfall.

Summary and Conclusions

The occurrence of *K. brevis* in Florida Gulf coast waters was significantly correlated with selected rainfall and flows

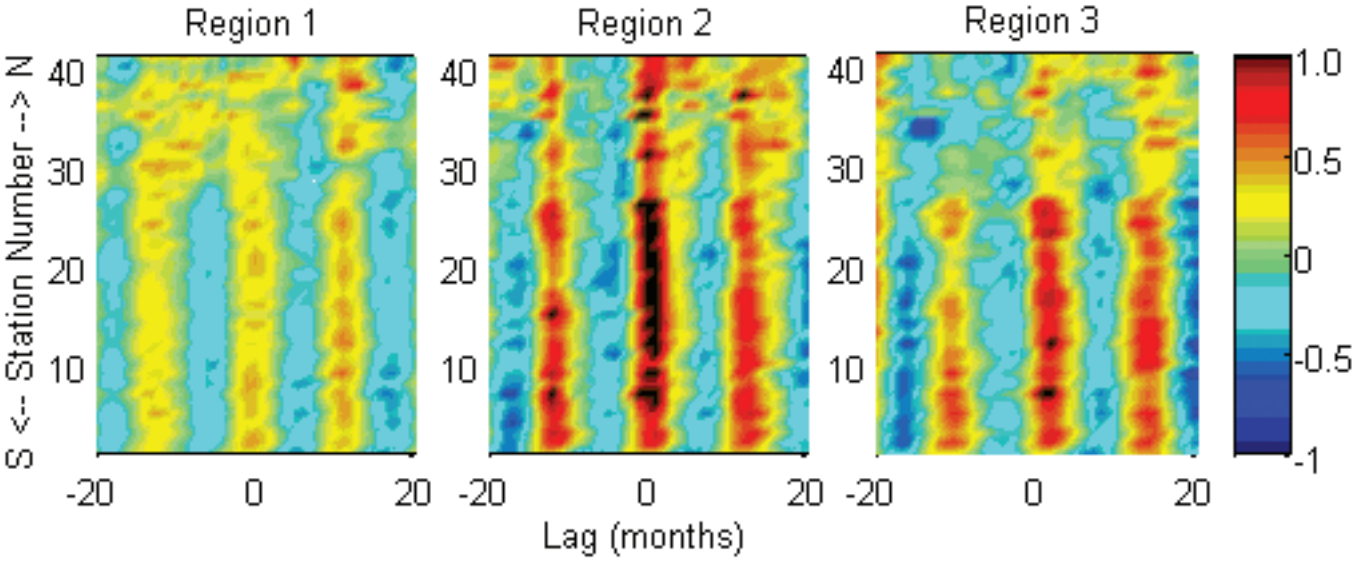

Figure 3 Geographically compiled, normalized cross-correlations of the three-month *K. brevis* duration parameter against monthly rainfall, by region. Values ≥1.0 (black) are significant at $P = 0.05$.

and most evident for the central region. Significant correlations were typically at short lag times. Geographic patterns were consistent in that significance generally declined with distance between Region and the location of the hydrologic parameter (rainfall or flow station). Patterns of significance varied between regions, indicating regional forcing functions. A scarcity of sampling in Region I, and short flow records or ungauged basins in the hydrologically complex south Florida limit the extent to which all pertinent hydrology can be linked with *K. brevis* in Regions I and III. The lack of significance for the largest discharge (Apalachicola River) or for highly managed systems (Caloosahatchee River) imply the importance of constituent load and delivery to coastal waters rather than discharge alone. Efforts are underway to estimate and evaluate the combined nutrient loads from Tampa Bay and Charlotte Harbor.

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Isotopic Constraints on Nutrient Sources Supporting the 2001 *Karenia brevis* Bloom

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Abstract

Blooms of the toxic red tide dinoflagellate *Karenia brevis* occur annually on the west Florida shelf (WFS), causing significant public health and economic problems. Because these blooms originate in oligotrophic offshore waters where inorganic nutrient concentrations are often at or below the limits of detection, their nutrient sources are enigmatic. The natural abundance stable isotopic signatures ($\delta^{15}N$ and $\delta^{13}C$) of particulate bloom biomass can provide important clues as to the nutrient sources sustaining *K. brevis* blooms. Particulate bloom samples from 1998, 1999 and 2000 were obtained in conjunction with the ECOHAB Florida project, and showed similar isotopic behavior when compared to values for the 2001 bloom. Average particulate $\delta^{15}N$ and $\delta^{13}C$ values within the 2001 bloom were 2.9 (+/– 1.4) ‰ and –19.4 (+/– 1.9) ‰, respectively. These values suggest that the bloom organisms were utilizing a nitrogen source with a relatively low $\delta^{15}N$ value and the presence of significant amounts of detrital carbon within the bloom. Because nitrate concentrations are not sufficient to sustain the high biomass typical of these blooms, some component(s) of the dissolved organic nitrogen (DON) pool may be important. Major sources of DON available on the WFS are regenerated nitrogen from N_2 fixation by *Trichodesmium* spp. that upwelled at the shelf break, regenerated nitrogen from diatom blooms, or DON released from floating seagrasses. The relatively low $\delta^{15}N$ signatures within the bloom suggest the importance of N_2 fixation with subsequent regeneration as a significant source.

Introduction

Blooms of the red tide dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve*) occur with an almost annual frequency off the west coast of Florida, with the region of highest bloom incidence extending from Tampa Bay south to Ft. Myers (Vargo *et al.,* 2001). This is a broad and shallow oligotrophic area of the WFS where inorganic N concentrations are often at or below detection limits (Heil *et al.,* 2001). Potential sources of nitrogen on the WFS include upwelled, benthic- and estuarine-derived DIN as well as several sources of dissolved organic nitrogen (DON). DON can derive from resuspended near-bottom diatom populations, decaying seagrasses or sargassum, or the N_2 fixing cyanobacterium *Trichodesmium.* Measured inorganic nitrogen concentrations within *K. brevis* blooms are insufficient to support high biomass, but concentrations of (DON) are significantly higher in this region. DON concentrations have been shown to range from 5 to 16 μM during the 1999 *K. brevis* bloom (Lester *et al.,* 2001). The presence of DON at these concentrations along with the ability of *K. brevis* to utilize organic N (Baden and Mende, 1978; Shimizu and Wrensford, 1993) suggests this pool as a potential nitrogen source.

Stable isotopic analysis of nutrient sources, particulate bloom biomass and associated dissolved material potentially provides a means to identify the nitrogen source supporting *K. brevis* populations. The relative natural abundance ratio of stable nitrogen isotopes (^{15}N, ^{14}N) has become an important tool in the study of the N sources supporting organic matter production in the marine environment (Sweeny and Kaplan 1980; Cifuentes *et al.,* 1988). Biological processes such as nitrification, denitrification, N_2 fixation, decomposition and remineralization can modify the isotopic signature of nitrogen source pools within an ecosystem (Smit 2001). With some knowledge of isotopic fractionation processes and typical isotopic values for available nutrient source pools, the isotopic signature of phytoplankton collected during *K. brevis* blooms can be used to infer a nitrogen source. Simultaneous analysis of carbon isotopes (^{13}C, ^{12}C) allows for further constraints to be placed on a specific N source.

We have investigated the N and C stable isotopic dynamics on the WFS during a large *K. brevis* bloom which occurred from Sept. to Dec. 2001 as well as the N stable isotopic dynamics in this region for blooms occurring in 1998, 1999 and 2000. Isotopic analysis of particulate material collected within and outside of bloom patches were used to evaluate possible N sources and to examine the spatial and temporal stable isotopic behavior of these blooms.

Materials and Methods

Particulate samples of bloom and non-bloom material were obtained during monthly ECOHAB cruises (Heil *et al.,* 2001) for 1998, 1999 and 2000 as well as the months of Sept., Nov. and Dec. 2001. October 2001 samples were obtained on a joint ECOHAB/NSF cruise in the same region. Sampling protocol for chlorophyll *a* (chl *a*), inorganic (NO_3^-, PO_4^{-2}, and NO_2^-) and organic nutrient concentrations, and concentrations of live *K. brevis* cells followed that of ECOHAB: Florida cruises (Heil *et al.,* 2001; Lester *et al.,* 2001). For isotopic analysis, duplicate water samples were filtered onto precombusted (450°C, 2 hr) Whatman GF/F filters and immediately placed in storage at 4°C. Filters were then lyophilized (24 hr) and analyzed with a Thermo Finnigan Delta plus XL continuous flow isotope ratio mass spectrometer. Differences in isotopic signatures are expressed relative to a standard, using DELTA (δ) notation: $\delta^{15}N$ or $\delta^{13}C$ ‰ vs. Std. = $[R_{sample}–R_{standard}]$ where R = atom % ^{15}N or ^{13}C.

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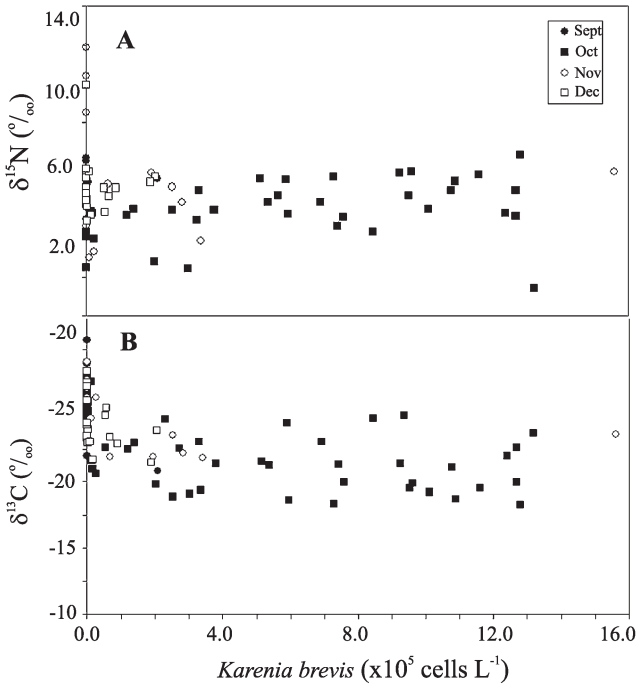

Figure 1 *Karenia brevis* concentration vs. **A** $\delta^{15}N$ and **B** $\delta^{13}C$ of particulate matter for all four months of the 2001 bloom.

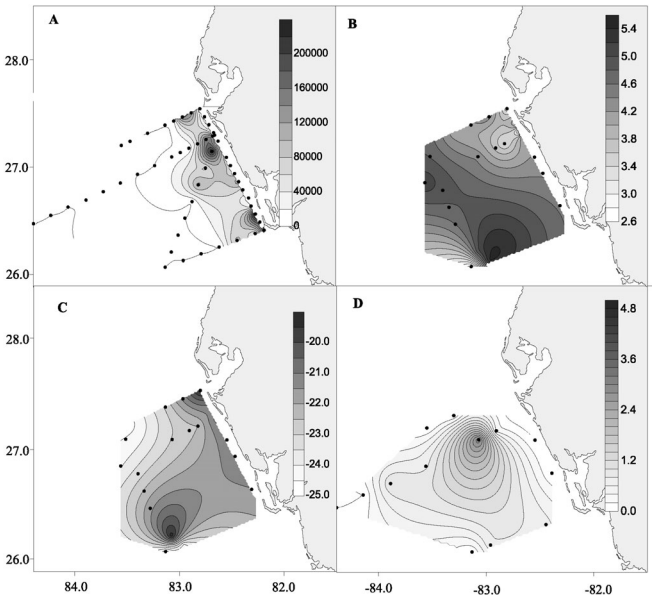

Figure 2 Surface plots of **A** *K. brevis* concentration (cells L^{-1}), **B** $\delta^{15}N$, **C** $\delta^{13}C$, and **D** *Trichodesmium* (colony L^{-1}) from Dec. 2001 ECOHAB: Florida cruise.

Results and Discussion

During the 2001 bloom, high *K. brevis* concentrations correlated with a restricted range of $\delta^{15}N$ (2–6‰) (Fig. 1A), suggesting the utilization of a constrained N source. High *K. brevis* concentration correlated with $\delta^{13}C$ values of ~ –20.5 to –17‰ (Fig. 1B), which are enriched relative to marine phytoplankton which typically show a range of –30 to –18‰ (Boutton, 1991). The enriched $\delta^{13}C$ of *K. brevis* during bloom events may be related to a decreased isotopic fractionation of DIC as a result of elevated growth rates, low ambient CO_2 concentration (Baird *et al.,* 2001) or the assimilation of DIC derived from macrophytes or heterotrophic processes.

Surface plots of $\delta^{15}N$ and *K. brevis* distribution from Dec. 2001 show a depleted $\delta^{15}N$ signature in the area of high bloom concentration (Fig. 2A, B), suggesting that blooms are utilizing an isotopically depleted nitrogen source. Surface plots of *Trichodesmium* spp. distribution during this month show depleted $\delta^{15}N$ signature in the area of high cell concentration (Fig. 2D). This reflects the ability of this cyanobacterium to fix N_2 (Glibert and Bronk, 1994), which has a $\delta^{15}N$ value of 0‰. Bacterial degradation, cell lysis and other processes contribute to the release of isotopically depleted DON from these cells. Available N derived from heterotrophic recycling of fixed atmospheric N_2 is potentially an important source of nitrogen sustaining large *K. brevis* blooms.

The Dec. 2001 surface plot of $\delta^{13}C$ exhibits an onshore to offshore gradient becoming more depleted offshore (Fig. 2C). This pattern may reflect a distinct isotopic signature of terrestrially derived material in this region (Boutton, 1991).

Average monthly ECOHAB $\delta^{15}N$ signatures for the 1998, 1999 and 2000 blooms show the same range of $\delta^{15}N$ values (2–~5‰) (Fig. 3) as seen in the 2001 bloom (Fig. 1), while showing more depleted values in between bloom periods. These are relatively depleted isotopic signatures for marine phytoplankton, which range up to 10‰ (Cloern *et al.,* 2002). Higher $\delta^{15}N$ signatures seen at areas of high bloom biomass (Table 1) may reflect decreased fractionation in accordance with the Rayleigh distillation model (Waser *et al.,* 1998).

The restricted ranges of isotopic signatures for both carbon and nitrogen within the large 2001 *K. brevis* bloom suggest that analysis of natural abundance stable isotopes is a powerful tool for the investigation of HAB dynamics.

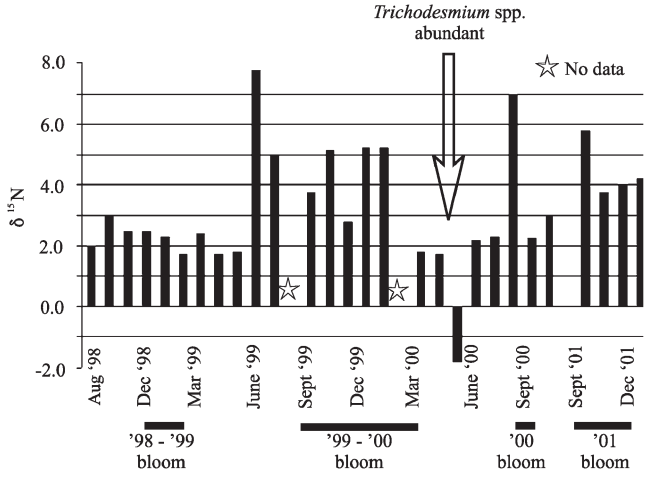

Figure 3 Average monthly $\delta^{15}N$ values of particulate matter collected during ECOHAB: Florida cruises. Bloom months are indicated by horizontal bars.

Table 1 $\delta^{15}N$ values (± standard error) of particulate material from stations where *K. brevis* concentrations > 50.000 cells L^{-1}.

Month	$\delta^{15}N$ (+ SE)
Dec 1998	4.88 (0.15)
Sept 1999	5.02 (0.40)
Oct 2000	5.11 (0.11)*
	3.63 (0.30)**
Sept 2001	5.02 (0.08)
Oct 2001	3.12 (0.36)
Nov 2001	4.20 (0.78)
Dec 2001	4.24 (0.27)

*Bloom 5 km off Sarasota **Bloom 20 km off Tampa Bay

Similarities between the $\delta^{15}N$ signatures of the 2001 bloom and the 1998, 1999 and 2000 blooms suggest that a common seasonal nitrogen supply exists for *K. brevis* blooms in the eastern Gulf of Mexico.

Acknowledgements

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Primary Productivity of Florida Red Tides

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Abstract

Primary productivity rates were measured at 2 stations along the Mote Marine Laboratory's red tide transect off Sarasota, FL, as well as in other west Florida coastal waters. Experiments have been performed almost monthly since June 2000 in West Florida Shelf coastal waters during red tides as well as during non-red tide periods. Productivity was estimated using the 14-carbon uptake method in an incubator utilizing ambient light and temperature conditions. Water samples are collected at 2 depths for each station. Cell counts of *Karenia brevis* varied from 0 to 18 million cells L^{-1} with chlorophyll *a* concentrations varying from <1 to 75 µg L^{-1}. Primary productivity rates averaged nearly 3 g C m^{-3} day^{-1}. This data will be used to supplement the current ECOHAB:Florida program. Establishing these stations at sites where red tides have occurred relatively frequently may yield the first productivity rates before, during and after a Florida red tide.

Introduction

Karenia brevis blooms occur along the central and southwest Florida coast nearly on an annual basis. They initiate offshore (18–74 km) on the mid West Florida Shelf and are most common during late summer and fall months (Steidinger *et al.,* 1998). The areal coverage and duration is quite variable (Vargo *et al.,* 1987) and patchiness is common. Phytoplankton primary production is a key component to understanding any community-level processes involving harmful algal blooms (HABs). HABs are normally associated with detrimental effects to ecosystems. However, Florida's red tide may contribute a substantial portion to the annual production of the West Florida Shelf (Vargo *et al.,* 1987). Primary productivity rates were measured at 2 stations along the Mote Marine Laboratory's red tide transect off Sarasota, FL, as well as in other coastal waters during a red tide.

Materials and Methods

Various stations were sampled for productivity experiments between 26°40′N and 26°44′N along the central, west Florida Coast out to approximately 15 nautical miles (Fig. 1). Water samples were collected at multiple depths for each station using either a CTD-rosette sampler or a Niskin-type sampler. Water was prescreened through a 153 µm mesh to remove larger zooplankton and then transported to the laboratory in dark containers for processing. Chlorophyll-*a* (chl *a*) concentrations were determined fluorometrically using a Turner fluorometer and a modification of the Welschmeyer (1994) technique. Cell concentrations of *Karenia brevis* were determined using light microscopy. Phytoplankton carbon assimilation, as an estimate of primary productivity, was determined using a modification of the method in Parsons *et al.* (1984). Productivity samples were incubated for 4 hours in a simulated *in-situ* incubator under natural sunlight conditions. Stainless steel screening was used to create 10 light levels (1–83% of PAR) in clear acrylic tubes through which flowing seawater maintained ambient temperatures. Photosynthesis-irradiance (PE) curves were generated using the equation from Platt *et al.* (1980): Eq. 1: $P^* = P^*_s (1–e^{-a})e^{-b}$ where $a = \alpha E/P^*_s$ and $b = ßE/P^*_s$. P* (mg C mg chl a^{-1} hr^{-1}) is the primary production rate normalized to chl *a*, P^*_s (mg C mg chl a^{-1} hr^{-1}) is the saturated rate of chl *a*-normalized photosynthesis in the absence of photoinhibition (ß), α [(mg C mg chl a^{-1} hr^{-1}) / (µEinsteins $m^{-2} \cdot s^{-1}$)] is the initial slope of the PE curve and E (µEinsteins m^{-2} s^{-1}) is the scalar irradiance. The maximum photosynthetic rate, P^*_m (same units as P^*_s) with a corresponding irradiance, E_m, can be derived from Eq. 1 and then the light saturation parameter, E_k (µEinsteins m^{-2} s^{-1}), can be calculated as $E_k = P^*_m / \alpha$. The daily integrated production (mg C m^{-2} day^{-1}) was calculated by multiplying the production (mg C m^{-3} day^{-1}) at the 47% light level by the water column depth.

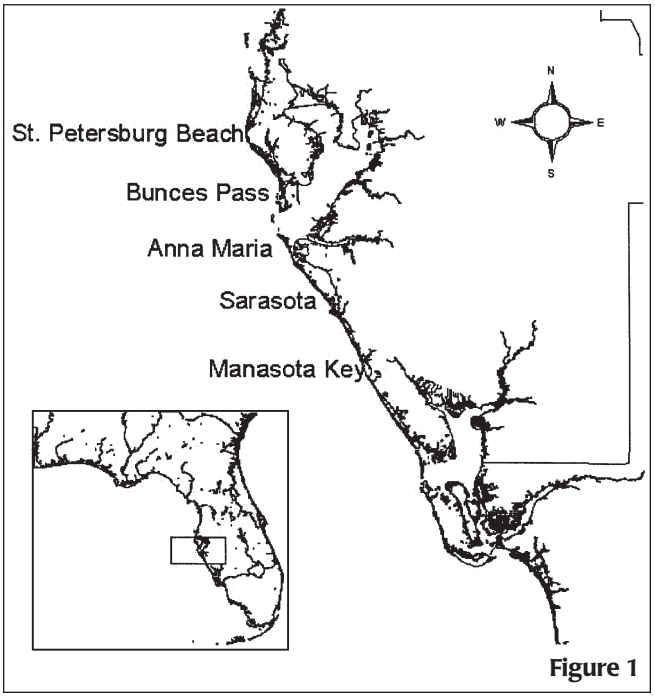

Figure 1

Steidinger, K. A., J. H. Landsberg, C. R. Tomas, and G. A. Vargo (Eds.). 2004. Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO.

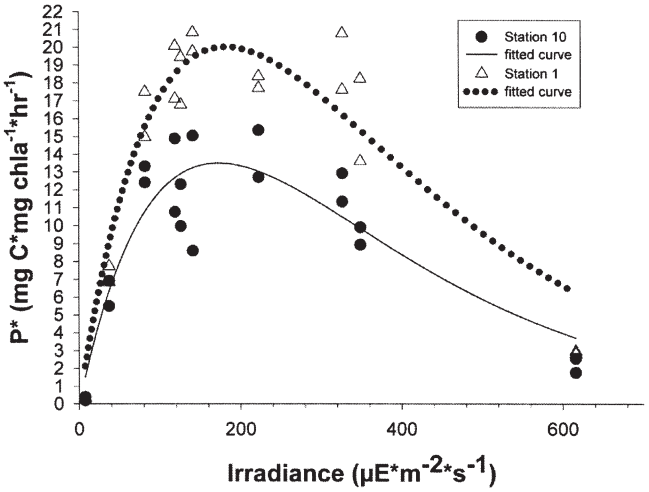

Figure 2 PE-curve for stations BP01 and BP10, 1 and 10 nm off Bunces Pass on 6/26/02.

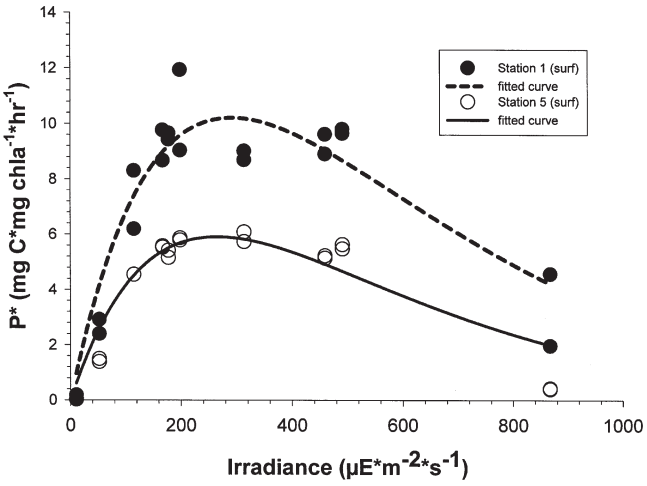

Figure 3 PE-curve for stations ECO01 and ECO5, 1 and 5 nm off Sarasota on 1/31/02.

Results and Discussion

A wide range of *Karenia brevis* cell concentrations (0–18.1 $\times 10^6$ cells L^{-1}) were sampled. Chl *a* concentrations varied from 0.28 to 75.5 (avg. = 10.72) µg L^{-1} with the highest values associated with high *K. brevis* cell counts and nearshore stations. Maximum photosynthetic rates, P_m, from 0.29–20.02 and an average of 6.80 mg C mg chl a^{-1} hr^{-1} were measured. Photosynthetic efficiencies, α, varied from 0.0061 to 0.2990; E_k varied from 35 to 147 (avg. = 74); E_m varied from 113 to 398 (avg = 204); and integrated production varied from 0.35 to 9.99 (avg = 345) (see Table 1).

Nearshore waters (BP01, ECO01) had higher P_m, chl *a*, α, and integrated production values than coastal water (ECO5, ECO10, BP5, BP10, BP15) (Table 1; Figs. 2, 3). Experiments from non-bloom stations (Fig. 4) showed higher P^*_m and α than those from stations with a *K. brevis* bloom (Fig. 5). Furthermore, photoinhibition occurred at higher light intensities in all experiments (Figs. 2–5). This result, coupled with the relatively low E_k and E_m values, indicate that these were shade-adapted populations. In a comparison of the photosynthetic parameters we measured to those measured by Shanley and Vargo (1993), the α, P^*_m and I_k values are relatively close considering the differing experimental conditions (lab vs. field), cell concentrations and physiological states (especially when the aberrant values from a red tide-free area on 6/26/02 are excluded). Shanley and Vargo (1993) measured (from 0.007 to 0.062 and P^*_m from 0.29 to 5.37 and Isat from 45 to 120. Production was greater in the red tide near Sarasota in October 2001 than in the red tide near Manasota Key in August 2001. Observations at that time indicated that the red tide moved north along the coast from south of Manasota Key to Sarasota during fall 2001 (FMRI and Mote Marine Lab, unpubl. data). Presumably, new sources of nutrients from Tampa and Sarasota

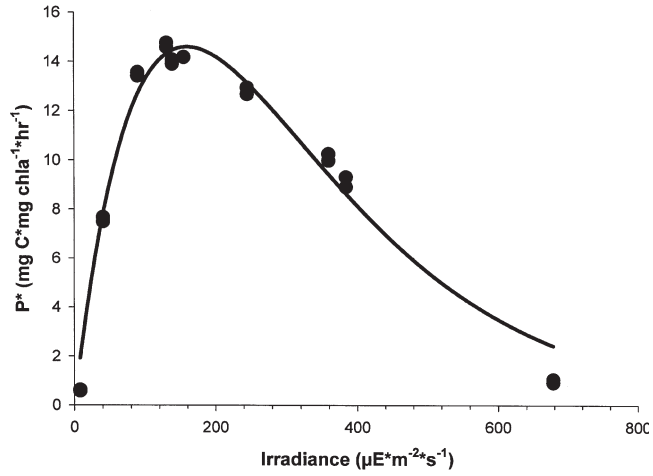

Figure 4 PE-curve from station 7 near Manasota Key on 8/30/01.

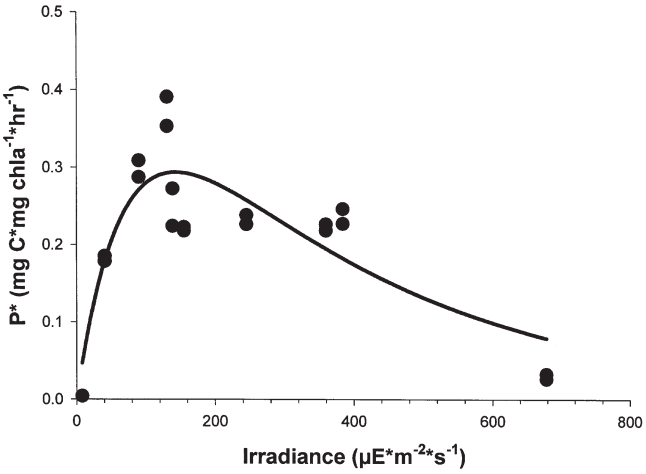

Figure 5 PE-curve from station 8 near Manasota Key on 8/30/01.

Table 1 Photosynthetic parameters, chlorophyll *a* concentration (μg L^{-1}), *Karenia brevis* counts (cells L^{-1}), and integrated production (mg C m^{-2} day^{-1}) for various stations along the west coast of Florida.

Date	Station	Location	Station Depth	Sample Depth	P_{max}	α	E_k	E_m	[Chl*a*]	*K. brevis*	Int prod
3/1/01	ECO01	Sarasota	10.6	0.77	10.42	0.0830	126	343	0.576	2000	665.42
3/1/01	ECO01	Sarasota	7.9	9.97	0.0840	118	321	1.88	0		
3/1/01	ECO10	Sarasota	15.5	0.86	7.55	0.0515	147	398	0.283	1000	349.45
3/1/01	ECO10	Sarasota	12.8	9.54	0.0733	130	354	0.684	0		
8/30/01	PPRT01	Manasota Key	7.7	0.5	0.43	0.0075	58.3	155.6	52.87	5,630,000	1272.75
8/30/01	PPRT07	Manasota Key	8.5	0.5	14.6	0.2480	58.8	160	2.04	0	2053.45
8/30/01	PPRT08	Manasota Key	4.6	0.5	0.29	0.0061	48	145	34.65	4,790,000	490.47
8/30/01	PPRT10	Manasota Key	2.3	0.5	1.28	0.0222	58.3	155.6	75.5	18,100,000	1708.37
9/5/01	BP01	Bunces Pass	6.1	0.5	13.06	0.1290	100	275	3.43	0	3770.09
9/5/01	BP05	Bunces Pass	9.6	0.5	10.31	0.1336	77	210	4.56	0	6348.27
9/5/01	BP10	Bunces Pass	11.2	0.5	4.13	0.0880	58	158	1.46	0	930.61
9/5/01	BP15	Bunces Pass	17.6	0.5	4	0.0545	73	199	0.68	0	838.34
10/3/01	ECO1	Sarasota	10.5	1	7.37	0.1303	57	155	12.44	9,000	9795.22
10/3/01	ECO5	Sarasota	12.5	1	5.85	0.0951	62	168	11.78	46,700	9988.39
10/3/01	ECO10	Sarasota	16.5	1	4.49	0.0879	57	154	8.41	30,000	6711.59
10/24/01	PB41	near Anna Maria	11	1.1	2.88	0.0631	44.4	125	10	600,000	2133.70
10/24/01	PB41	near Anna Maria	11	6	4.02	0.0855	47.2	127.8	9.02	630,000	
10/24/01	PB41	near Anna Maria	11	9.4	1.46	0.0302	47.2	130.6	9.16	367,000	
11/15/01	MML dock	Sarasota	2	surf	3.65	0.1032	35	155	11	500,000	1049.64
11/15/01	ECO01	Sarasota	9	surf	3.44	0.0903	37	113	5.27	233,000	2157.36
1/31/02	ECO01	Sarasota	9.5	1.4	10.21	0.0952	107	291	2.4	10,700	2661.36
1/31/02	ECO5	Sarasota	11	1.2	5.9	0.0606	97	265	2.84	100,000	2080.79
1/31/02	ECO5	Sarasota	11	8	4.63	0.0549	84	229	5.04	333,000	
5/31/02	Culture	St. Pete	3.81	0.0639	60	162	10.97	843,000			
6/26/02	BP01	Bunces Pass	6.5	surf	20.02	0.2990	67	182	1.35	0	1954.14
6/26/02	BP10	Bunces Pass	11.8	surf	13.49	0.2123	64	173	0.47	0	731.20

Bays may have "regenerated" the bloom, thereby allowing for greater production values. The red tide off Manasota Key had lower production than other blooms and even other nearshore waters when a red tide was not present, *e.g.,* Bunces Pass.

The estimated production rates that we have measured are near those given in Vargo *et al.* (1987), but our average of nearly 3 g C m^{-2} day^{-1} is double theirs. The chl *a* concentrations that we encountered are also greater. Therefore, we may have measured productivities in more dense blooms, which would result in higher rates and indicate that red tides contribute significantly more carbon to the shelf system than previously thought.

Acknowledgements

We wish to thank the staff of Mote Marine Laboratory for use of their vessel, R/V *Eugenie Clark,* to collect samples. Thanks also to Gabe Vargo at USF for providing light data and Earnest Truby for providing *K. brevis* cell counts and the map. Funding for this project was provided by the ECOHAB program, NOAA grant NA06OP0530.

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Bacterial and Size Fractionated Primary Production Within a Large *Karenia brevis* Bloom on the West Florida Shelf

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Abstract

In October 2001, bacterial and size-fractionated primary productivity was measured within and outside a large *Karenia brevis* bloom on the west Florida shelf (WFS). Primary production in the >0.2 µm fraction within the bloom ranged from 19.2 to 127.3 mg C m^{-3} hr^{-1}, while depth integrated values ranged from 2.9 to 14.3 mg C m^{-2} d^{-1}. An average of 52.3 (±18.8)% of production occurred in the >3.0 µm fraction and could be attributed directly to *K. brevis.* Smaller *Karenia* cells (*i.e.,* gamete-like cells, recently divided or amitotically produced cells) composed up to 32.2% of total *Karenia* abundance within the bloom and possibly accounted for significant production in the 0.4–3.0 µm fraction. Outside the bloom, rates of primary production within oligotrophic surface water, the deep chlorophyll *a* maximum and *Trichodesmium* populations were 1.76, 5.29 and 2.64 mg C m^{-3} d^{-1}, respectively. Concurrent bacterial productivity measurements were an order of magnitude higher inside the bloom than outside, and rates of bacterial productivity were about twofold higher in whole water than in <1.2 µm water. These results suggest that bacteria were associated with particles within the bloom or that larger *Karenia* cells were competing heterotrophically for leucine.

Introduction

Episodic blooms of the toxic dinoflagellate *Karenia brevis* contribute significantly to annual production on the west Florida shelf (Vargo *et al.,* 1987). During blooms, rapid recycling of organic and inorganic nutrients may enhance production by other phytoplankton and bacteria; however little is known about associated bacterial and primary productivity within or near blooms. *K. brevis* does not grow axenically in culture, but *K. brevis* specific algicidal bacteria have been reported (Doucette *et al.,* 1999), which suggests that bacteria may serve a complex role in this species ecology. Evans (1973) formalized a working hypothesis (based in part on work by Bein, 1954) in which bacteria have 2 potential roles in *K. brevis* blooms: 1) as a passive cause of red water discoloration and 2) as an active stimulator as a supplier of nutrients (*e.g.,* vitamins). Although high concentrations of bacteria have been reported from *K. brevis* blooms in 1972, 1982, 1987 (Evans, 1973; Buck and Pierce, 1989), no measurements of bacterial productivity from *K. brevis* blooms have been made to date.

In 2001, the west Florida shelf experienced a large *K. brevis* bloom which persisted for >13 months. Size-fractionated bacterial and primary production was measured during peak bloom biomass (Fig. 1) to 1) establish bacterial and primary productivity within an unusually large bloom relative, and 2) determine the size fraction responsible for production within the bloom.

Materials and Methods

Primary and bacterial production was measured by $H[^{14}C]O_3$ and [^{3}H]Leucine incorporation according to Parsons *et al.* (1984) and Kirchman (1993), respectively. Water samples were taken with Nisken bottles, gently siphoned into sample bottles, and incubated in on-deck incubators continuously flushed with surface water between 1000 and 1700 hr. Light attenuation was provided by neutral density screening and bacterial samples were incubated in darkness. Size fractionated uptake was determined at termination of incubations by sample filtration onto 0.45, 1.0 or 3.0 µm nucleopore filters. All samples were counted with a TM Analytic Delta 300 Liquid Scintillation Counter. Bacterial controls consisted of sample with 5% TCA additions, and leucine biomass production estimates assumed 1) a fraction of leucine in protein of 0.073, 2) a cellular C/Protein ratio of 0.86 and 3) an isotope dilution of 2.

Cell concentrations were determined from gluteraldehyde (3%) preserved samples using a Sedgewick-Rafter Chamber and an Olympus BH-2 microscope. *Karenia* species were differentiated on the basis of cell morphology and nuclear placement. "Small" cells of *Karenia* could not be identified to species but were differentiated as vegetative or gamete-like cells based on cell morphology. Size measurements were made during enumeration using an ocular scale bar calibrated with an Olympus micrometer.

Results and Discussion

The 2001–2002 *K. brevis* bloom was characterized by the longest duration (>13 months) and highest biomass (chl *a* >10 µg L^{-1}) of the 4 blooms sampled during the ECOHAB:Florida program. Primary production rates in the >0.2 µm fraction within the *K. brevis* bloom in September ranged from 19.2–127.3 mg C m^{-3} hr^{-1}, with depth integrated values ranged from 2.9–14.3 mg C m^{-2} d^{-1} (Table 1). These values are more than double the rates reported by Vargo *et al.* (1987) and an order of magnitude greater than values from outside of the bloom. These results suggest that blooms of *K. brevis* of the magnitude and duration which occurred in the 2001–2002 season, although relatively rare (Steidinger,

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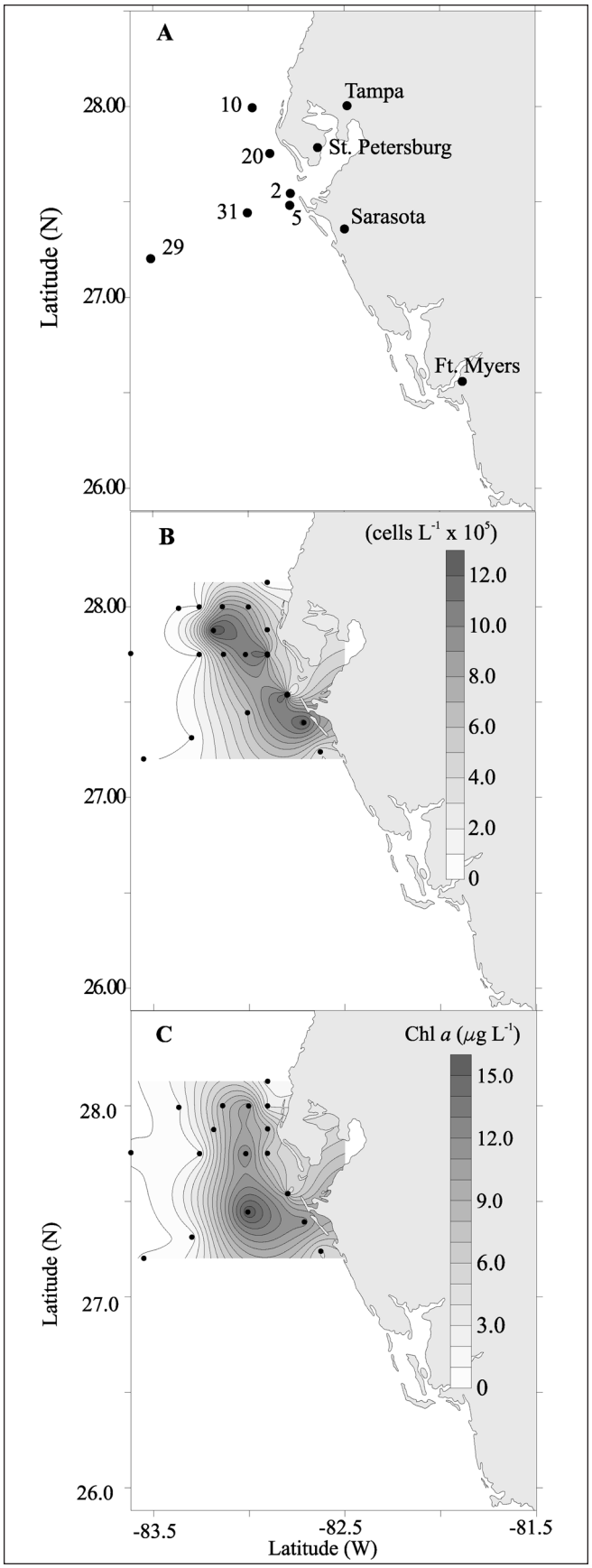

Figure 1 **A** Locations of stations sampled for production, **B** surface *K. brevis* concentrations, and **C** surface chl *a* concentrations.

Table 1 Primary and bacterial biomass production rates within and outside the 2001 *K. brevis* bloom. (NM: Not measured).

	Bacterial Production (ng C L^{-1} h^{-1})		Primary Production (mgC m^{-3} h)
	0.45–1.2 μm	>1.2 μm	>0.2 μm
Within Bloom	13.1–48.7	0.0–88.4	19.2–127.3
Outside Bloom (St. 29)			
Oligotrophic	1.25	2.3	0.68–1.76
Deep Chl Max	NM	NM	1.84–5.29
Trichodesmium spp.*	NM	NM	2.64

*Calculated from measured *Trichodesmium* abundance at st. 29.

pers. comm.), may contribute more to production and nutrient cycling on the WFS than previously thought. Only 42–75% of primary production within the bloom could be attributed to large vegetative *Karenia* cells, however, as significant production was present in the 0.4–3.0 μm fraction within the bloom (Fig. 2). Flagellates and small diatoms composed a relatively small proportion of the total phytoplankton population compared with *Karenia* cells at all bloom stations (Table 2), suggesting they may have been responsible for only a small percentage of this production in the <3.0 μm fraction. Microflagellates may have contributed significantly; however, an alternative explanation is that this <3.0 μm production was attributable to "small" cells of *K. brevis* and *K. mikimotoi.* "Small" vegetative cells (*i.e.,* gamete-like cells, recently divided cells and smaller vegetative cells resulting from amitosis) are common in dinoflagellate genera (Silva and Faust, 1995) and have been reported for both *K. brevis* and *K. mikimotoi* (Wilson, 1957; Partensky and Vaulot, 1989; Partensky *et al.,* 1991). Within the bloom, they composed up to 32.2% of total *Karenia* cells (Table 2). Although the measured length and width of "small" *Karenia* cells was greater than the 3.0 μm filter size cut-off (Table

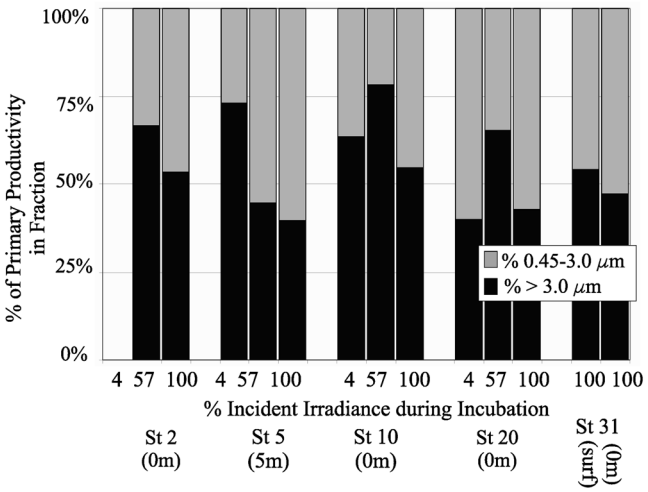

Figure 2 Relative proportions of primary production associated with 2 size fractions within the 2001 *K. brevis* bloom.

Table 2 *Karenia,* total diatoms and flagellate concentrations at productivity stations, with the percentage (%) of the *Karenia* population present as "small" cells.

Station	Depth (m)	Concentration (cells L^{-1}* 10^5)				% of *Karenia* as "small" cells
		K. brevis	*K. mikimotoi*	Diatoms	Flagellates	
2	0	6.41	0.45	3.71	1.09	12.87
5	0	3.35	0.27	0.27	1.51	32.21
10	0	11.10	0.09	0.06	0.17	9.59
20	0	7.42	0.10	0.04	0.19	14.79
29	0	0.00	0.00	0.01	0.05	–
31	Surface**	18.08	0.17	0.52	0.46	7.63
	0	10.39	0.08	0.04	0.57	9.96

*Includes gametes, newly divided cells and possibly "small" vegetative cells. **sample taken from surface bucket.

Table 3 Size measurements of numerically abundant *Karenia* species and cell types in the 2001 bloom. All units in µm (+SD).

	Cell Type	Length	Range	Width	Range	N
K. brevis	"Large"*	25.4 (3.2)	22.0–29.7	30.1 (4.0)	24.2–35.2	15
K. mikimotoi	"Large"*	26.1 (2.9)	23.1–31.9	23.7 (14.0)	22.0–26.4	7
Karenia spp.	"Small"*	14.0 (2.8)	11.0–19.8	13.51 (2.9)	9.9–18.7	14
Karenia spp.	Gamete-like	14.0 (0.6)	13.2–14.3	10.18 (1.0)	8.8–11.0	4

*Refers to vegetative cell types.

3), *Karenia* cells are morphologically plastic. Larger *Karenia* vegetative cells can pass through 5.0 µm Nitex screening (Vargo, pers. comm.) and under gentle filtration, it is likely that "small" vegetative *Karenia* cells were able to pass through 3.0 µm filters.

Bacterial biomass production was significantly higher within the *K. brevis* bloom compared with outside the bloom (Table 1). This observation supports the hypothesis that significant *Karenia* bacterial interactions occur within the bloom, possibly related to nutrient recycling. In addition, significant amounts of ^{3}H-leucine incorporation during the bloom were associated with particles >1.2 µm (Fig. 3). This suggests 2 possibilities: 1) that significant amounts of bacteria were associated with particles within the bloom and/or 2) that larger cells were competing heterotrophically for leucine. Shimizu and Wrensford (1993) also report uptake of ^{13}C-leucine by *K. brevis* in culture and Bronk *et al.* (these proceedings) demonstrated uptake of urea and glutamate by *K. brevis* during this 2001 bloom, which supports the role of organic nitrogen sources in contributing to *K. brevis* blooms.

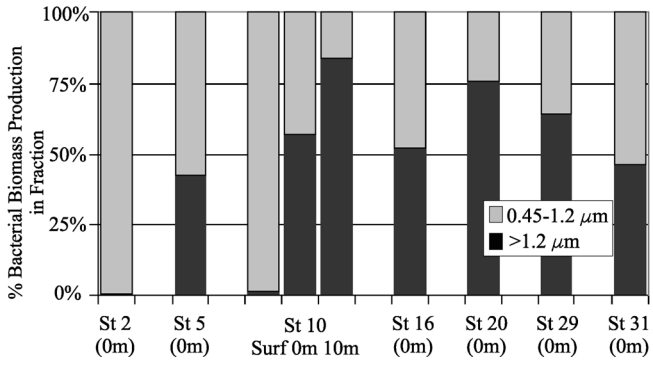

Figure 3 Relative proportions of ^{3}H-Leucine incorporation into 2 size fractions inside and outside the 2001 *K. brevis* bloom.

Acknowledgements

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Harmful Algal Blooms in the Western Gulf of Mexico: *Karenia brevis* Is Messin' with Texas and Mexico!

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Abstract

The eastern Gulf of Mexico is beset with *Karenia brevis,* a toxic dinoflagellate that blooms nearly annually causing neurotoxic shellfish poisoning, massive fish kills, marine mammal deaths and significant losses to local economies. Less well understood is the fact that *K. brevis* frequently occurs elsewhere in the Gulf of Mexico, most notably in the western Gulf. Over the past seven years (1996–2002) *K. brevis* blooms have been reported every year except 1998 in this region. During the *K. brevis* bloom in 2000, all the bays along the Texas coast, including Galveston Bay, had *K. brevis* cell counts high enough to close shellfish waters for the first time.

Introduction

In Texas coastal waters and bays, *Karenia brevis* bloom activity, reported as fish kills, has been recorded sporadically since the mid 1800s (Kusek *et al.,* 1999). The frequency and co-occurrence of harmful blooms in Texas and the Mexican states of Veracruz and Tamaulipas are not widely recognized. In at least one of these Mexican states, fish kills associated with *K. brevis* blooms have occurred in the same general time periods as blooms in Texas. Co-occurrent blooms have been documented from the mid 1940s forward (Table 1). Alongshore transport mechanisms may be involved in moving the blooms from one area to another, but the details of this process are currently undocumented. Most *K. brevis* blooms in the Eastern Gulf of Mexico are known to initiate offshore (Tester and Steidinger 1997). Villareal *et al.* (2001) suggest that *K. brevis* blooms along the Texas coast initiate >15 km offshore and are transported into the near shore areas. Along the Texas coast there are many high salinity bays and lagoons where *K. brevis* blooms can become established. Recent observations indicate that residual *K. brevis* populations may persist in Texas bays and lagoons following major coastal bloom events. Remnant populations could initiate recurrent blooms and present unique management problems.

Table 1 Frequency of *Karenia brevis* blooms in the western Gulf of Mexico.

MEXICO	TEXAS
	1935 [a]
1946 [b]	1946–1947 [b]
1955 [c]	1955 [c]
1974 [d]	1974 [d]
	1986 [e]
1994–1995 [f,g]	
1996 [f,g]	1996 [h,i]
1997 [f,g]	1997 [h,j]
1999 [g]	1999 [i,j]
2000 [g,k]	2000 [j]
2001–2002 [k]	2001–2002 [k]

Sources of information: [a] Lund, 1936; [b]Gunter *et al.,* 1948; [c] Wilson and Ray, 1956; [d] Buskey 1996; [e]Trebatoski, 1988; [f] Sierra-Beltran *et al.,* 1998; [g] Marea Roja Foro Nacional, 2002; [h] Texas Dept. Health, 1997; [i] Texas Parks and Wildlife, 2000; [j] Texas Dept. Health, 2000; [k] Texas Dept. Health, 2001–2002.

Materials and Methods

Karenia brevis cell counts from the Texas Department of Health were compiled from 1996 through 2002 and entered into a geographic information system database (ArcView 3.2 ESRI™). Toxin data were provided by the Servicios de Salud de Veracruz and were compiled using landmarks associated with the station descriptions.

Results and Discussion

Increasing frequency of *K. brevis* blooms in western Gulf of Mexico are causing unprecedented closures of shell fishing areas (Table 1). For the first time (December 2001), all Mexican shellfishing was suspended because of harmful algal blooms along both the Pacific and Gulf coasts. Following this event, the 1er Marea Roja Foro Nacional was held February 2002 in Mexico City. A National Plan was developed under the auspices of the Directorate for Environmental Health.

One of the most extensive *K. brevis* blooms in the western Gulf of Mexico occurred from late August 2000 to early February 2001 (Fig. 1). There were reports of "red water" in every bay or estuary along the Texas coast, with maximum cell counts exceeding 37,000 cells mL^{-1} in San Antonio Bay. In Galveston Bay, a major shellfishing area in Texas, *K. brevis* cell numbers were as high as 18,000 cell mL^{-1}, resulting in the closure of shellfish beds (Fig. 2). Harvesting was suspended for up to four months in some areas. This represented the first time Galveston Bay has been closed to shellfishing because of a toxic phytoplankton bloom.

Once *K. brevis* becomes established in the high salinity

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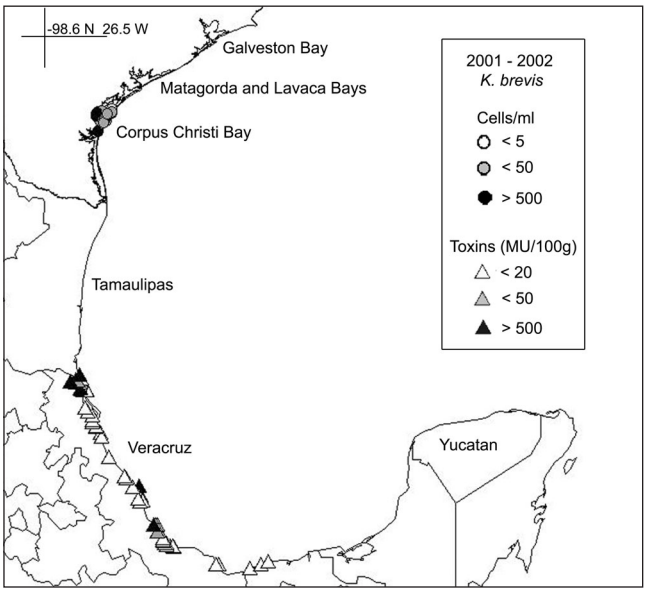

Figure 1 Distribution and abundance of *Karenia brevis* cells in Texas and toxicity (brevetoxin measured in mouse units $100g^{-1}$ shellfish tissue) in Mexico during the 2000–2001 bloom. In *K. brevis,* toxicity is related to cell counts such that the toxicity values represent relative cell densities.

bays of the western Gulf, they present an interesting management issue. While most *K. brevis* blooms initiate offshore (Tester and Steidinger, 1997), remnant blooms in Texas bays or lagoons may be source regions for localized blooms. In Florida, the low-salinity, upper reaches of the bays do not support *K. brevis* growth because it is a neritic species with a salinity requirement of >24 psu (Aldrich and Wilson, 1960). The data from Lavaca Bay (a secondary bay inland from Matagorda Bay, Texas) provides an example of where density stratification may have maintained higher numbers of 4 cells in the bottom waters while the surface

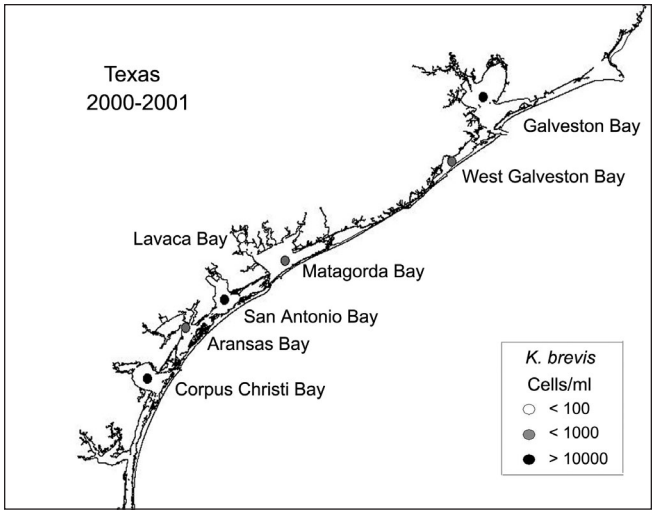

Figure 2 Distribution and maximum cell counts of *Karenia brevis* in the major Texas bays and estuaries during the 2000–2001 bloom.

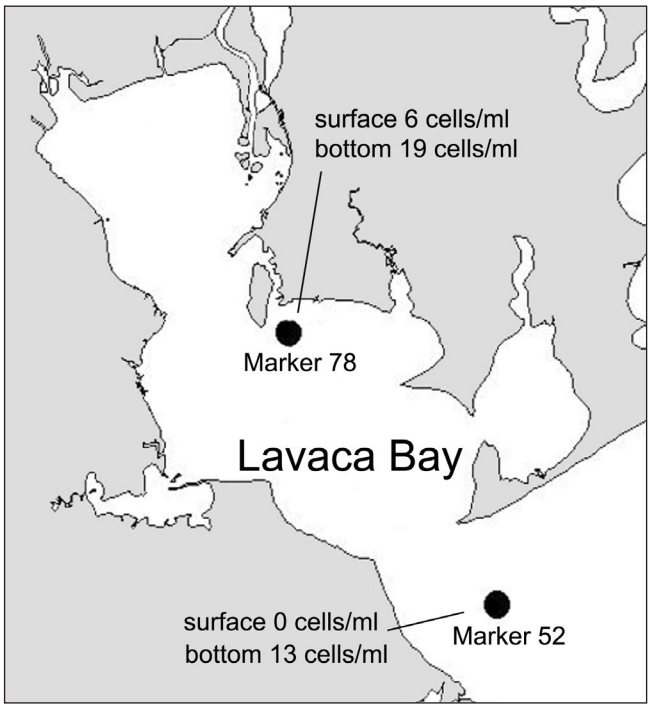

Figure 3 Lavaca Bay, Texas, where *Karenia brevis* cell counts are at or below closure levels in surface waters and three- to fourfold closure limits in bottom waters.

waters had few or no cells (Fig. 3). There are a number of times when shellfish beds have been closed because of a short-lived *K. brevis* bloom that was not associated with an offshore bloom or widely noted (see Buskey, 1996). If the sub-surface cells persist in backwater areas where flushing is infrequent, these cells may serve to initiate blooms. This necessitates continued monitoring throughout the entire water column until cell numbers are below 5 cells mL^{-1}, even after the bloom has dissipated in the main portion of the bay.

Summary

Combined *Karenia brevis* cell counts and brevetoxin data from Texas and Mexico indicate that *K. brevis* blooms may be occurring more frequently in the western Gulf of Mexico and that remnant blooms of this species may be established in poorly flushed, high salinity estuaries along the coast of Texas. With heightened economic and public health concerns in both Mexico and Texas, never has there been a more opportune time for collaborative efforts between the two countries to determine the conditions that initiate, transport and foster *K. brevis* blooms.

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Effects of the Toxic Red Tide Dinoflagellate (*Karenia brevis*) on Survival, Fecal Pellet Production and Fecundity of the Copepod *Acartia tonsa*

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Abstract

The effects of the toxic red tide *Karenia brevis,* on the survival, egg production, hatching success and fecal pellet production of the copepod *Acartia tonsa* were studied. The same measurements were made for *A. tonsa* fed the similarly sized dinoflagellate *Scrippsiella trochoidea,* the diatom *Thalassiosira* sp. and under starvation conditions. Results indicate that while *K. brevis* is not directly toxic to *A. tonsa,* it is not utilized as a food item. Grazing rates on *K. brevis* were close to zero and almost no differences existed between survivorship, behavior and egg production of *A. tonsa* in the starved and *K. brevis* treatments. Both *S. trochoidea* and *Thalassiosira* sp. were readily grazed and utilized as food items.

Introduction

Toxic effects of dinoflagellates on crustacean zooplankton include reduced survival, loss of neuromuscular control, elevated heart rate, reduced feeding, reduced egg production, and deleterious effects on developmental stages (Gill and Harris, 1987; Huntley *et al.,* 1986; Turner and Tester, 1997). Zooplankton have been known to avoid waters containing bloom levels of dinoflagellates (Fiedler, 1982; Huntley, 1982). In some cases copepods can selectively feed on non-toxic algae during blooms of toxic algae (Huntley, 1982; Ives, 1987; Turriff *et al.,* 1995), in other cases the presence of toxic algae can interfere with the feeding on non-toxic algae (Huntley *et al.,* 1986). Several studies have shown that copepods are capable of consuming some species of toxic alga (Uye, 1986; Ives, 1987; Uye and Takamatsu 1990; Turriff *et al.,* 1995; Turner and Tester, 1997). By grazing toxic phytoplankton the zooplankton can be a toxic link to higher trophic levels (White, 1981; Teegarden and Cembella, 1996). Not all grazers can consume the same toxic alga without harmful effects (Uye and Takamatsu, 1990; Teegarden and Cembella 1996; Turner and Tester, 1997). It has been suggested that past exposure of the population to the harmful algal species may be necessary for toxin resistance to be present (Shumway and Cucci, 1987). Thus, contradictory results may occur as different populations of the same species of grazer might show different reactions to the same species of toxic algae. *Acartia tonsa* is the most abundant copepod species in Texas waters (Buskey, 1993). This study examined the effects of the red tide dinoflagellate *K. brevis* on *A. tonsa* compared to the effects of a similarly sized dinoflagellate *Scrippsiella trochoidea,* a diatom *Thalassiosira* sp. and starvation conditions. Effects of these diets on copepod survival, egg production, hatching success, and fecal pellet production were measured.

Materials and Methods

Zooplankton were collected with a 153 μm mesh, 0.5 m diameter plankton net from a pier in Port Aransas, Texas and live *A. tonsa* were sorted from the collected zooplankton. The dinoflagellates *K. brevis* (clone SP-3) and *S. trochoidea* were cultured in L-1 media while the diatom *Thalassiosira* sp. was cultured in f/2 media. All phytoplankton were cultured at 20°C on a 12-hour light/dark cycle. For the survival experiments, ten adult females were placed into each treatment. Three treatments were each run in triplicate, *Thalassiosira* sp. (1 mg C l^{-1}), *K. brevis* (1 mg C l^{-1}) and 0.20 mm filtered seawater. The *A. tonsa* were held in 250 mL of treatment culture in covered 10.5 cm diameter Carolina culture dishes at 20°C on a 12 hour light-dark cycle. Each treatment was examined under a dissection microscope every 24 hours for six days. Egg and fecal pellet production of *Acartia tonsa* was examined for different feeding regimes with 1.0 mg C l^{-1} food: *Thalassiosira* sp., *K. brevis, S. trochoidea,* a mixture of *S. trochoidea* and *K. brevis* and without food (0.20 μm filtered seawater). Ten adult female and one adult male *A. tonsa* were placed in each treatment. Treatments were prepared in triplicate in one-liter polycarbonate bottles, placed on a plankton wheel (ca. 1 rotation min^{-1}), in an environmental chamber on a 12 hour dark/light cycle and held at a constant temperature of 21°C. The copepods were allowed to graze for 24 hours, screened through nested 153 μm and 40 μm mesh sieves. The adult *A. tonsa* were retained on the 153 μm mesh, while eggs and nauplii were retained on the 40 μm mesh and food items passed through both. The adult *A. tonsa* were returned to the screened incubation water. After an additional 24 hours the cultures were screened again and egg and fecal pellet production were measured by direct counting under a dissection scope. For egg-hatching-success experiments, 20 eggs produced from a laboratory incubation of ten adult females and one adult male in 1.0 mg C l^{-1} *Thalassiosira* sp., *K. brevis, S. trochoidea,* or a mixture of *S. trochoidea* and *K. brevis* were placed in petri dishes for hatching. The eggs were hatched in the same phytoplankton cultures used for the egg production incubations. After 24 hours the nauplii were counted to obtain egg-hatching success rates.

Results

The survival experiment demonstrated that *A. tonsa* fed *Thalassiosira* sp. had higher survivorship than either those fed *K. brevis* or those starved (Fig. 1). Survivorship was sig-

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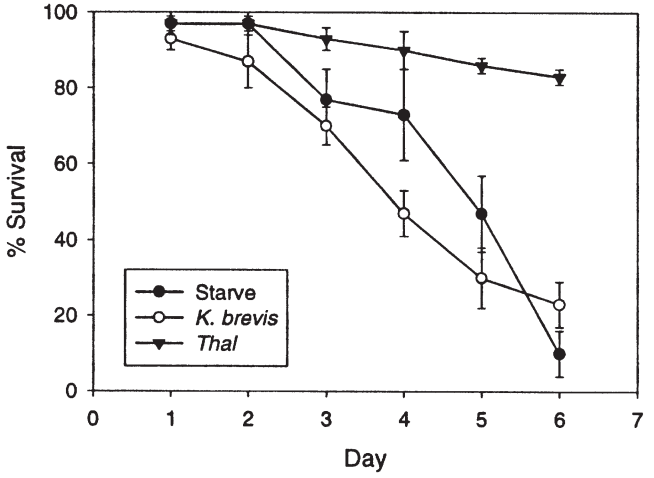

Figure 1 Survivorship of *Acartia tonsa* cultured in 1 mg C l^{-1} of *Thalassiosira* sp. (*Thal*), *K. brevis* (*K. brevis*), and 0.20 µm filtered seawater (Starve). Error bars are one standard error.

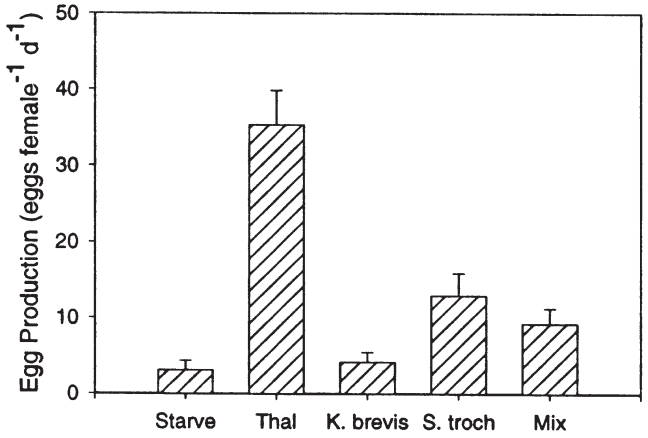

Figure 2 Egg production female^{-1} day^{-1} of *Acartia tonsa* cultured in 1 mg C l^{-1} *Thalassiosira* sp. (*Thal*), *K. brevis, S. trochoidea* (*S. troch*), a mixture of *S. trochoidea* and *K. brevis* (Mix) or 0.2 µm filtered seawater (Starve). Error bars are one standard error.

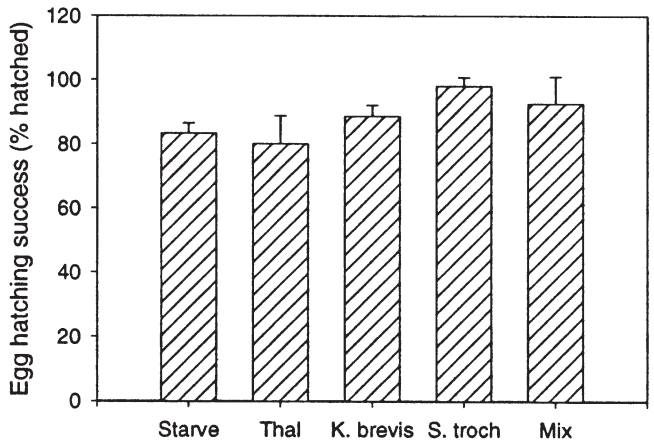

Figure 3 Hatching success of eggs produced by parents reared in 0.20 µm filtered seawater (Starve) on 1 mg C l^{-1} of *Thalassiosira* sp. (*Thal*), *K. brevis, S. trochoidea* (*S. troch*) and a mixture of *K. brevis* and *S. trochoidea* (Mix). Error bars are one standard error.

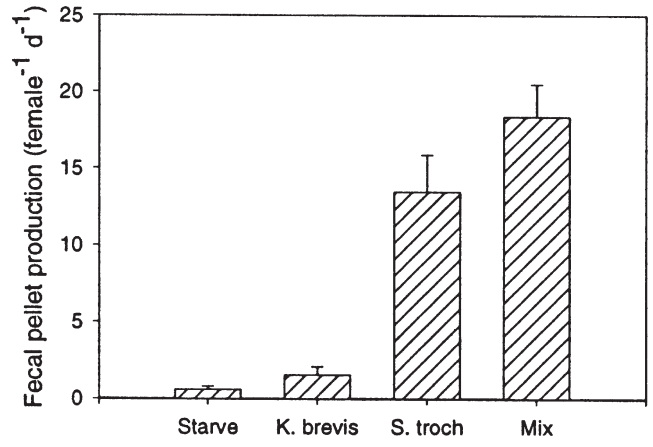

Figure 4 Fecal pellets female^{-1} day^{-1} of *A. tonsa* cultured in 1 mg C l^{-1} *K. brevis, S. trochoidea* (*S. troch*), a mixture of *S. trochoidea* and *K. brevis* (Mix) and 0.2 µm filtered seawater (Starve). Error bars are one standard error.

nificantly lower ($P < 0.05$) in the *K. brevis* treatment than the *Thalassiosira* sp. treatment for day 4 through day 6 and in the starvation treatment than the *Thalassiosira* sp. treatment for day 5 and day 6. Survivorship was never significantly different between the starvation and *K. brevis* treatments. For the egg production experiment, *A. tonsa* fed *Thalassiosira* sp. had a significantly higher egg production rate than they had in any of the other treatments (Fig. 2). Egg production was not significantly different in the starved and *K. brevis* treatments. *Acartia tonsa* fed *S. trochoidea* had higher egg production rates than those fed *K. brevis* or starved. No significant difference was found in egg production of the *A. tonsa* fed the mixture and those fed *S. trochoidea* alone. Hatching success of *Acartia tonsa* was 80% or higher in all treatments (Fig. 3). Hatching success of the eggs produced and hatched in the *S. trochoidea* treatment was significantly higher than those in the starved treatment. No other significant differences were found between the various treatments.

Fecal pellet production was significantly higher when *Acartia tonsa* was fed *S. trochoidea* or the mixture than when fed *K. brevis* or starved. No significant difference was found in fecal pellet production of the starved *A. tonsa* and the *A. tonsa* fed *K. brevis* (Fig. 4).

Discussion

The survivorship study indicates that although it is not readily ingested, *K. brevis* is not directly toxic to *A. tonsa* (Fig. 1). The presence of *K. brevis* in the water is not acutely toxic to *A. tonsa,* as *A. tonsa* survivorship rates are not significantly different in the starvation treatments and the *K. brevis* treatments over a six-day period. The fecal pellet

production (Fig. 4) experiments clearly demonstrate that *K. brevis* is not consumed by *A. tonsa. Acartia tonsa* fed *K. brevis* produced almost no fecal pellets.

The lowered egg production of *K. brevis*-fed *A. tonsa* (Fig. 2) indicates that either *K. brevis* is not consumed or it is an extremely poor food item. The relatively high egg production when fed the similarly sized dinoflagellate *S. trochoidea* demonstrates that algal size does not interfere with consumption or utilization, and that *A. tonsa* can feed well on other species of dinoflagellates. The similarly high values of egg production when *A. tonsa* were fed a mixture of *K. brevis* and *S. trochoidea* reveal that unlike in some red tide studies (Huntley *et al.,* 1986) *K. brevis* does not appear to interfere with the feeding of another food source (*S. trochoidea*) beyond direct particle interference. The high *A. tonsa* egg production in the *K. brevis* and *S. trochoidea* mix as well as the high egg hatching success in *K. brevis* treatments (Fig. 3) also suggests that *K. brevis* does not have any direct toxic effects on *A. tonsa.*

If grazing rates are high enough and grazers numerous enough, zooplankton grazing can have a significant effect on an algal bloom. Watras *et al.* (1985) found that zooplankton grazing rates on *Alexandrium tamarense* blooms can exceed the population growth. Even if grazing rates are relatively low, zooplankton grazing pressure could be important in retarding the initial stages of bloom development (Uye, 1986). However, since *A. tonsa* does not consume *K. brevis,* but is not prevented from consuming other food items, *A. tonsa* could aid in the initiation and maintenance of a *K. brevis* bloom. By grazing down the other phytoplankton species and possible microzooplankton grazers, *A. tonsa* can bring about a reduction in resource competition and predation on *K. brevis.*

Although the presence of *K. brevis* is clearly toxic to fishes, it appears to have minimal or no direct toxic effects on *A. tonsa.* Results of this study clearly show that while *K. brevis* is not utilized as a food item by *A. tonsa,* it does not lead to a significantly higher death rate than starvation. These studies have shown that the presence of *K. brevis* does not prevent *A. tonsa* from feeding on any suitable food item that is available. However, since *A. tonsa* does not feed upon *K. brevis* it would not be a factor in grazing down a bloom. In fact it is possible that *A. tonsa* could aid the initiation or continuation of a bloom by grazing down competitors or microzooplankton grazers of *K. brevis.*

Acknowledgements

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Does Nitrogen Regeneration from the N_2 Fixing Cyanobacteria *Trichodesmium* spp. Fuel *Karenia brevis* Blooms in the Gulf of Mexico?

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Abstract

Trichodesmium alleviate nitrogen (N) limitation where they occur by using atmospheric dinitrogen (N_2). Much of the recently fixed N_2 is regenerated as ammonium (NH_4+) and dissolved organic N (DON). This regenerated N is then available to support the growth of other cells. We hypothesized that N regenerated from N_2 fixation provides the N necessary to support blooms of *Karenia brevis* in the Gulf of Mexico, and have conducted a combination of field and laboratory investigations to demonstrate a viable nutritional link and to quantitatively assess the role of *Trichodesmium* in providing N to support the growth of *K. brevis.* Results demonstrate that *Trichodesmium* fix N_2 at high rates with more than 50% of this new N released as NH_4+ and DON and that *K. brevis* has a high affinity for reduced N sources. In addition to these indirect lines of evidence, we have conducted a number of studies to establish direct links between *Trichodesmium* and other primary producers. In the field, stable isotopes were used to trace the uptake of $^{15}N_2$, its regeneration into dissolved N and its subsequent uptake into plankton biomass. Dialysis bags containing *Trichodesmium* were suspended in gas-tight incubation bottles containing *K. brevis* and $^{15}N_2$ enriched water. We observed that regenerated ^{15}N label (as NH_4+ and DON) passed through the dialysis bags and was taken up by phytoplankton outside of the bags. With these experiments we demonstrate that N released from *Trichodesmium* can support the growth of associated phytoplankton and possibly *K. brevis*.

Introduction

Blooms of the toxic dinoflagellate, *Karenia brevis,* occur in the oligotrophic waters of the eastern Gulf of Mexico, where known nitrogen (N) sources are insufficient to support observed biomass accumulations (Steidinger *et al.,* 1998, Walsh and Steidinger, 2001). For example, estuarine transport could supply just 5% to 20% of the N required for the daily growth needs of a moderate population (3×10^5 cells/L) of *K. brevis* (Vargo *et al.,* this Proceedings). However, it has been observed that large *K. brevis* blooms frequently co-occur or occur subsequent to blooms of the N_2-fixing cyanobacteria *Trichodesmium* sp. (Walsh and Steidinger, 2001), and correlations between the timing and magnitude of blooms of *Trichodesmium* sp. and *K. brevis* in the Gulf of Mexico and the coastal Atlantic have been made based on historical red tide monitoring data, unpublished research results and anecdotal information (Lenes *et al.,* 2001). *Trichodesmium* sp. fix dinitrogen (N_2) at high rates and thereby bring new N and contribute to new production in otherwise nutrient depleted waters (Capone *et al.,* 1997).

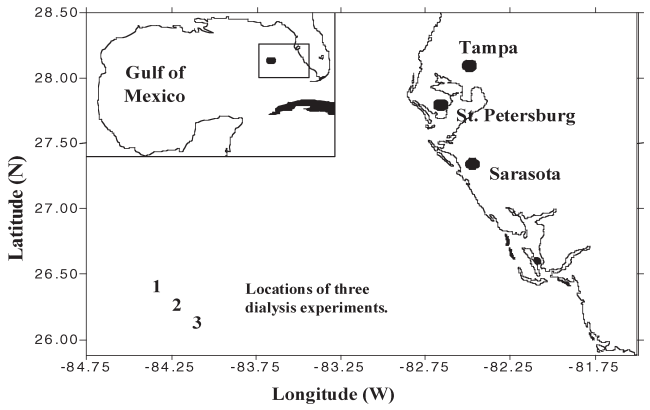

Figure 1 The Gulf of Mexico study area off the West Florida Shelf. DOTGOM #1 Cruise, R/V *Walton Smith,* July 7–13, 2001.

It has also been observed that *Trichodesmium* release fixed N as dissolved organic N (DON) (Glibert and Bronk, 1994), amino acids (Capone *et al.,* 1994) and ammonium (NH_4^+) (Mulholland and Capone, 2001; Mulholland *et al.,* unpublished results) into the dissolved pool where it is available for uptake by other organisms. Accumulation of NH_4^+ and/or DON has been observed in and around *Trichodesmium* blooms in the Pacific (Karl *et al.,* 1992, 1997; Devassy, 1987), the Gulf of Mexico (Lenes *et al.,* 2001, Heil *et al.,* unpublished data), along the coast of Australia (O'Neil *et al.,* submitted) and in aging cultures (Mulholland and Capone, 2001). The objective of this study was to determine whether release of dissolved N from N_2 fixation is sufficient to fuel observed red tide blooms in the Gulf of Mexico. To do this we first needed to quantify rates of N_2 fixation by *Trichodesmium* in the Gulf of Mexico and then determine the ecological fate of the recently fixed N_2.

Methods

To achieve our objectives, we measured the following: (1) N_2 fixation by *Trichodesmium*, (2) the production of dissolved N by natural populations of *Trichodesmium*, (3) N uptake by *K. brevis,* and (4) the transfer of regenerated N to co-occurring plankton during cruises in the Gulf of Mexico aboard the R/V *Walton Smith* in July 2001 and aboard the R/V *Suncoaster* in October 2001 (Fig. 1). On the July cruise, a drogue was deployed and used to follow a sur-

Steidinger, K. A., J. H. Landsberg, C. R. Tomas, and G. A. Vargo (Eds.). 2004. Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO.

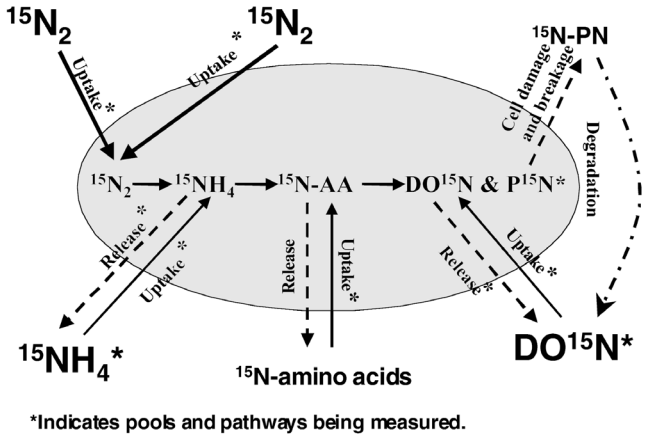

Figure 2 Pools of dissolved and cellular N and pathways for uptake, metabolism and release.

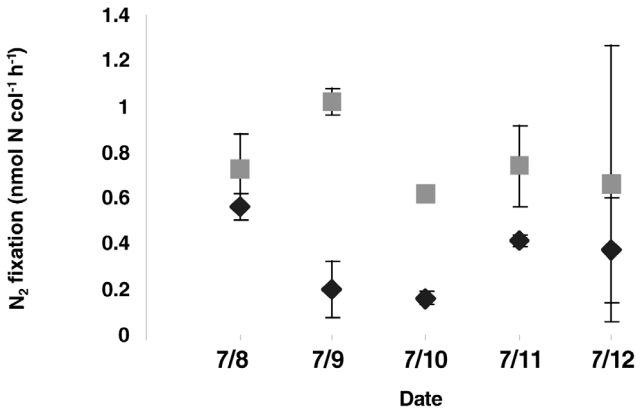

Figure 3 Gross N_2 fixation measured using acetylene reductions (squares) and net $^{15}N_2$ uptake (diamonds) during a cruise aboard the R/V *Walton Smith* in July 2001.

face population of *Trichodesmium* sp. for 5 days. *Trichodesmium* colonies were collected from the upper 20 m using a 202-µm, 1-m plankton net towed at speeds <1 knot. Colonies were transferred to filtered (GF/F, nominal pore size of 0.8 µm) seawater using inoculating loops and then transferred to experimental incubation bottles. We measured gross N_2 fixation by colonies of *Trichodesmium* using the acetylene reduction technique (Capone, 1993). In addition, highly enriched $^{15}N_2$ tracer was used to follow the transfer of N among cellular (particulate) and dissolved pools (after isolating dissolved NH_4^+ and DON) (Fig. 2). In a separate set of experiments, we contained *Trichodesmium* colonies in dialysis tubing with either 1 kDalton or 100 kDalton size cut-offs and suspended the sealed tubes in bottles containing natural plankton communities without *Trichodesmium*. We added $^{15}N_2$ and after 2 hours, measured uptake into *Trichodesmium* and into the surrounding plankton. We also incubated controls where we added $^{15}N_2$ to natural populations without *Trichodesmium*-containing dialysis bags. During one of these experiments, we added copepods that are known to graze on *Trichodesmium* to the dialysis bags to see if this would increase the production of dissolved N compounds. During the October cruise, kinetic studies were done to estimate N uptake by *K. brevis* (Bronk *et al.,* this Proceedings)

Results and Discussion

Rates of N_2 fixation ranged from 0.62 to 1.02 nmol N col^{-1} h^{-1} over the 5-day drogue study using the acetylene reduction method (Fig. 3). However, when we measured $^{15}N_2$ uptake, net rates of N incorporation were much lower, ranging from 0.16 to 0.56 nmol N col^{-1} h^{-1}, than gross N_2 fixation measured using acetylene reduction. Production rates of $^{15}NH_4^+$ (0.3–1.2 pmol col^{-1} h^{-1}) and DO^{15}N compounds from $^{15}N_2$ were also low, and we believe this is because our incubation times were short (2 hours) and *Trichodesmium* colonies had substantial intracellular metabolite pools. Intercellular pools ranged from 0.58 to 3.42 nmol NH4+ col^{-1} in an earlier study (Mulholland and Capone, 1999). The time required to fill these pools with newly fixed ^{15}N would have been at least an hour, and this would have resulted in the initial release of unlabeled compounds rather than recently fixed $^{15}N_2$. Based on the difference between rates of N_2 fixation estimated from acetylene reduction and net $^{15}N_2$ uptake, we estimate N release rates ranging from 0.16 to 0.82 nmol N col^{-1} h^{-1}, or 22% to 80% of gross N_2 fixation.

To put this into the context of *K. brevis'* N demand, *Trichodesmium* abundance was between 0.6–7.8 col L^{-1} during our 5-day study, and this would result in a release of about 0.1 to 6.4 nmol N L^{-1} h^{-1}. While background concentrations of *Trichodesmium* are about 0.75 col L^{-1}, summer *Trichodesmium* concentrations are generally much higher, averaging 20 col L^{-1}. During blooms, densities along the west Florida shelf can reach >1000 col L^{-1} (Heil, unpublished data). Based on rates of N uptake by *K. brevis* (Bronk *et al.,* this Proceedings), we calculate an N demand on the order of 0.15 to 1.15 nmol N L^{-1} h^{-1}. This is well within the range of N release rates measured during our July study. Independently, Vargo *et al.,* (this Proceedings) calculated a total N demand of 0.056 to 0.267 mmol L^{-1} d^{-1} for a moderate (3×10^5 cells 10^{-1}) *K. brevis* population undergoing 0.2 divisions d^{-1}. Again, if we assume 10 hours of N_2 fixation per day and that *Trichodesmium* blooms are of similar duration and magnitude, during the July cruise the release rates of dissolved N were 0.001 to 0.064 mmol L^{-1} d^{-1}. If we invoke the higher *Trichodesmium* densities observed during the summer and during blooms, *Trichodesmium* can produce ample dissolved N to fuel *K. brevis* population growth.

For dialysis bag experiments, uptake of released ^{15}N-compounds that passed through the <1 kDalton bags, was always greater than the controls to which no dialysis bag containing *Trichodesmium* was added. The appearance of label in the surrounding plankton was due to NH_4^+ or DON regeneration and subsequent re-incorporation (Fig. 4). Up to 2% of the recently fixed $^{15}N_2$ was taken up by whole water. Uptake of dissolved material <100 kDalton was 2 to 22 times greater than uptake of the <1 kDalton material indicating significant utilization of higher molecular

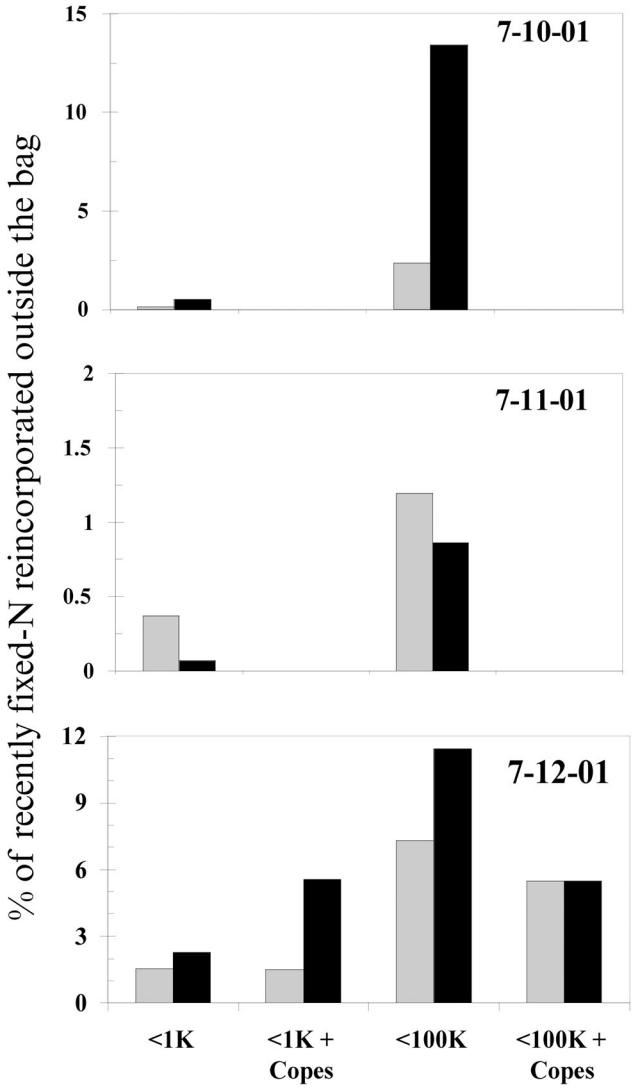

Figure 4 Percent of N_2 fixed by *Trichodesmium* contained in dialysis bags that was incorporated by plankton outside of the dialysis bags. The two bars are replicates. Controls had no $^{15}N_2$ uptake and are not shown.

weight DON. Up to 11% of the recently fixed N_2 was reincorporated by cells in whole water. Unexpectedly, uptake of recently released nitrogen did not increase when copepods (*Miracia efferata*) known to feed on *Trichodesmium* were added to the dialysis bags. We consider these reincorporation rates to be underestimates because we consider the production of dissolved N from $^{15}N_2$ to be underestimated due to the problem of large intracellular metabolite pools (see above).

We conclude that recently fixed N_2 from *Trichodesmium* is released as dissolved N and can be taken up by natural populations of plankton. N_2 fixation and subsequent DON and NH_4^+ release is a significant source of nitrogen to the plankton, including *K. brevis,* in the eastern Gulf of Mexico. This is the first step toward demonstrating a direct link between N_2 fixation by *Trichodesmium* and the harmful bloom-former *K. brevis.*

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Status of *Pfiesteria* Science, Including Tests of *Pfiesteria shumwayae* Strain CCMP2089 for Ichthyotoxicity and Toxin

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Abstract

It has been well established that there can be high strain variation in toxicity within toxic algal species, including *Pfiesteria piscicida* and *P. shumwayae*. Research by several laboratories with many strains has indicated that *Pfiesteria* spp. can kill fish with toxin involvement when cultured under conditions that allow active toxicity expression. Here we tested *P. shumwayae* strain CCMP2089, which was reported by other laboratories as nontoxic with fish prey when grown under conditions that may have suppressed active toxicity in this species. We conducted standardized fish bioassays, then re-cloned the strain and grew it in pure culture with only axenic cryptomonad algal prey for 6 weeks. Cultures were analyzed for toxin in blind tests. Strain CCMP2089 produced a toxin that both killed fish and was cytotoxic to rat pituitary cells (GH4C1 cell line). Both fish-fed and algal-fed (pure) cultures of CCMP2089, as well as positive controls (known toxin-producing strains of *P. piscicida* and *P. shumwayae*), contained the same chromatographic and biological properties from this water-soluble *Pfiesteria* toxin not found in negative controls (fish without dinoflagellates, fish with similar densities of cryptoperidiniopsoids as "pedunculate controls," or cryptomonads alone; trace amounts in non-inducible *Pfiesteria* cultures). There was 100% fish survival in all negative controls, and 100% death of fish along with confirmation of the presence of this *Pfiesteria* toxin in both water and cells of positive controls and of CCMP2089. This study shows the importance of using culture techniques that allow toxicity expression in *Pfiesteria,* the importance of appropriate analyses for toxin, and the importance of using multiple strains in tests for toxicity at the species or genus level.

Introduction

The fundamental toxicity of *Pfiesteria* spp. was negated by two recent, widely publicized papers (Berry *et al.,* 2002, Vogelbein *et al.,* 2002) that based their conclusion of "no *Pfiesteria* toxicity" on tests with 1 strain of *Pfiesteria shumwayae* (CCMP2089, Center for Culture of Marine Phytoplankton, Boothbay Harbor, ME). Both papers asserted that the strain had been grown under conditions used in standardized fish bioassays (SFBs) to culture actively toxic *Pfiesteria* (TOX-A functional type; Burkholder *et al.,* 2001a). In reality, however, SFB conditions had not been followed to obtain potentially toxic inocula for the micro-assays of Vogelbein *et al.* (2002) nor for the attempts to detect toxin by Berry *et al.* (2002); as examples, the pH was as low as 5.6; reactive NH_3 plateaued at ca. 30 mg/L; and in general, as the authors stated in their referenced methods paper, water quality was seriously degraded. In contrast, SFBs to ensure *Pfiesteria* spp. toxicity expression should maintain pH at >6.8, total NH_3 at <1 mg/L, and other conditions indicative of good water quality (Burkholder *et al.,* 2001a).

Most ichthyotoxic strains tested by our laboratories through 2000 lost capability for active ichthyotoxicity after weeks to months without live fish (Burkholder *et al.,* 2001b). Several strains, however, regained toxicity 1.0–1.5 yr after separation from live fish and were actively ichthyotoxic for up to 4 yr in culture (Burkholder *et al.,* 2001b); more recently, many toxic strains have been maintained for >2 yr. Here we tested strain CCMP2089 to determine whether it was toxic or non-inducible (= unable to kill fish with toxin involvement; Burkholder *et al.,* 2001a). We hypothesized that this strain would be capable of toxicity to fish when grown under conducive conditions in SFBs, or when first grown in SFBs and then re-cloned and grown for several weeks in pure culture with algal prey (the latter, based on recent research by NOS-Charleston and Springer *et al.,* 2002).

Materials and Methods

Strain CCMP2089 had been grown at the CCMP on cryptomonad algal prey for ca. 2 yr. It was cultured by NCSU, ODU and USC coauthors, in biohazard III facilities with juvenile tilapia in SFBs (15 psu, made with artificial seawater salts + ultra-filtered deionized water, then re-cloned and grown with axenic cryptomonad algal prey (~10 algal prey/ dinoflagellate at 2- to 3-day intervals for 6 weeks; n = 3) as in Burkholder *et al.* (2001a–c). SFBs were monitored for water quality parameters as in Burkholder *et al.* (2001a; except here, daily for pH). The algal-fed cultures were included to verify that the toxin detected from SFBs, which would have also contained other microbes associated with fish, came from *Pfiesteria*. We compared the data to results for comparable densities of known toxin-producing *P. piscicida* and *P. shumwayae* strains (tested as capable of killing larval fish ± physical contact) fed fish vs. algal prey (n = 3).

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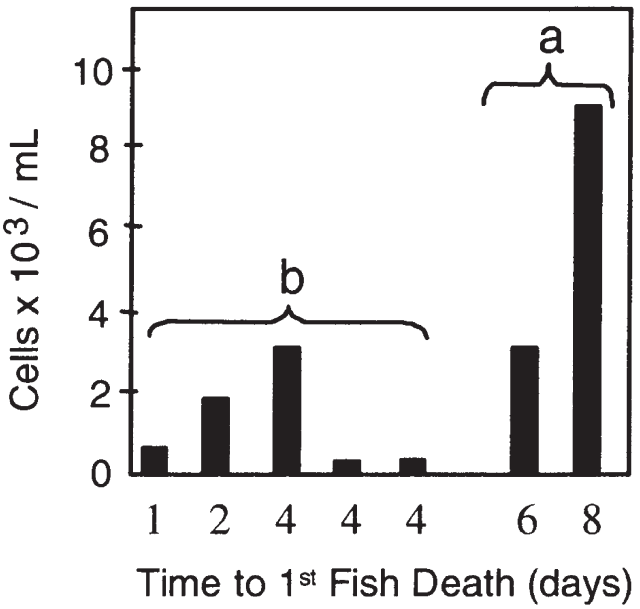

Figure 1 Time to first fish death for strain CCMP2089 in standardized fish bioassays (SFB)s: **a** Two subcultures were taken directly into SFBs from the original algal-fed clone (initial inoculum 200–450 cells/mL); **b** Subsamples from 1 of the 2 subcultures, which had required 8 days (at 3000–8800 cells/mL) to first kill juvenile tilapia, were transferred into 5 additional SFBs (initial density 400–2900 cells/mL) to test time to first fish death with prior killing history. These subcultures resumed killing activity faster (in 1–4 days) at significantly lower cell densities (400–2000 cells/mL; $P < 0.05$) There was no fish death, nor signs of stress, in the negative controls, whereas all fish died in positive controls (data not shown).

Negative controls included fish treated identically but without dinoflagellates; fish with comparable densities of cryptoperidiniopsoid dinoflagellates or NON-IND *Pfiesteria* (200–2000 cells/mL; n = 3); and cryptomonad algal prey alone. Fish-fed and algal-fed subcultures reached ca. 3×10^3–10^4 cells/mL. Cultures were processed for toxin extraction, isolation and analysis for GH_4C_1 cytotoxicity following Moeller *et al.* (2001). The partially purified toxin was also tested for larval fish response (sheepshead minnow, *Cyprinodon variegates,* age 7 days; in 5-mL Petri dishes containing 15-psu ultra-filtered seawater, 0.22 µm porosity; n = 5; controls given the same concentration solvent carrier without toxin). The first chromatographic isolation step partitioned the toxic extracts using a normal phase process that was carried out on beaded silica because of the need for use with higher water content to elute polar metabolites. This chromatographic method, using an elutropic solvent scheme of increasing polarity, partitioned the soluble extract derived from *Pfiesteria* culture into 3 fractions: 100% ethyl acetate (EtOAc), followed by 100% methanol (MeOH), and 1:1 methanol : water.

Results and Discussion

When strain CCMP2089 was grown in SFBs (consistently at pH >7, ammonia <1 mg/L, and other standard conditions), it killed fish fairly rapidly (Fig. 1). For the first 2 subcultures, time to first fish death was 6–8 days at densities ranging from 360–8,000 cells/mL. There was no significant correlation between initial or final cell densities and time to first fish death ($P > 0.05$). As dead fish were repeatedly replaced with live fish, the time to fish death in SFBs decreased to ≥4 hr. All negative control fish remained healthy; all positive control fish died in ≥4 hr.

The cytotoxicity assays coupled with sequential chromatographic analyses indicated that the fish-fed and pure, algal-fed CCMP2089 cultures, fractionated on silica with MeOH, contained a water-soluble toxicity that did not differ from that derived from *Pfiesteria piscicida*. Highest toxic activity was detected from the 100% MeOH fraction eluted from the silica column. Activity was lower from the 1:1 methanol : water fraction, likely because some toxin remained on the column. LC-MS retention times (Fig. 2) and all of the major ions in the mass spectra in the active fractions did not differ for the toxin isolated from *P. shumwayae* CCMP2089 and *Pfiesteria piscicida,* whether grown on fish or algal prey. No lipophilic cytotoxicity was observed from the EtOAc fraction in these experiments, although contaminating phthalate esters occasionally have given positive results on the GH_4C_1 cytotoxicity test (Moeller *et al.,* 2001). Negative controls did not yield this toxic activity, except for trace amounts from NON-IND *P. piscicida*. All larval fish exposed to *Pfiesteria* toxin (20 µL, extracted from 15 L of 4,800 cells/mL) died in <5 min, whereas all control fish given the same concentration of solvent carrier without toxin remained healthy.

Pfiesteria spp. had earlier been shown to attack fish by both physical and chemical mechanisms (Burkholder and Glasgow, 1997; Burkholder *et al.,* 2001a,b). Both toxic and nontoxic strains are capable of causing larval fish death by physical attack in microassays at moderate to high densities (10^3–10^4 cells/mL; Burkholder *et al.,* 2001a). For example, in one study TOX-A strains caused larval fish death in <6 hr on average; TOX-B strains killed larval fish in ca. 15 hr; and NON-IND strains killed in ca. 24 hr at densities of 10^4 cells/mL (Burkholder *et al.,* 2001a). Highly toxic strains have been shown to be capable of causing death of larval finfish and shellfish, and of juvenile and adult finfish in standardized fish bioassays (Burkholder *et al.,* 2001a, with confirmation of the presence of the water-soluble *Pfiesteria* toxin), in minutes to several hours (Burkholder and Glasgow, 1997; Springer *et al.,* 2002). Some toxic *Pfiesteria* strains can kill fish without physical contact (Burkholder and Glasgow, 1997; Gordon *et al.,* 2000; Springer *et al.,* 2002). Others have required close proximity to fish prey or direct contact, and in all cases, fish death was more rapid when physical contact has been allowed (Burkholder and Glasgow, 1997; Gordon *et al.,* 2000; Burkholder *et al.,* 2001c; Springer *et al.,* 2002). In contrast, although some NON-IND strains have been able to kill low numbers of larval fish slowly, in repeated SFBs they have not been able to kill juvenile or adult fish at moderate to high cell densities (Burkholder *et al.,* 2001b).

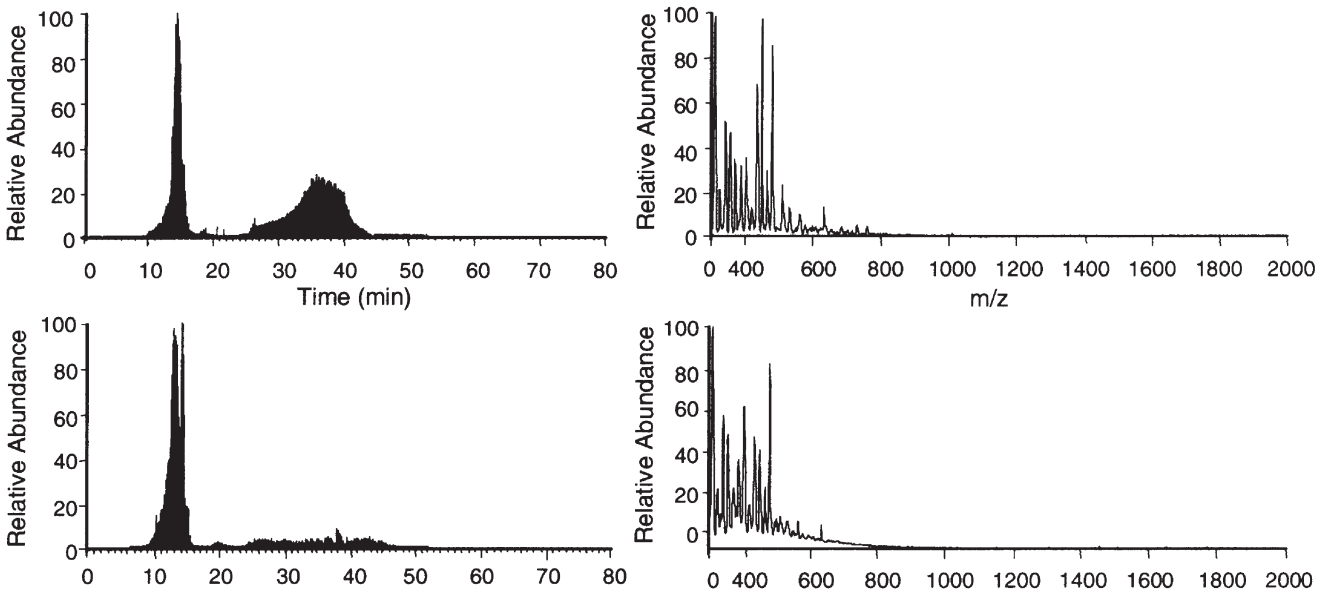

Figure 2 HPLC-MS chromatograms (left panels) and LC-MS spectra (right panels) for the chromatographic peak of interest for extracted toxin from *P. shumwayae* CCMP2089 (top panels) and *P. piscicida* (bottom panels). Major ions from both spectra and peak numbers directly corresponded. This toxin was both ichthyotoxic and cytotoxic to mammalian cells (rat pituitary GH4C1 cell line; also see Levin *et al.,* 2003).

P. shumwayae strain CCMP2089 was confirmed to be both ichthyotoxic and cytotoxic to rat pituitary cells when cultured under conditions known to allow active toxicity expression. The data support previous research indicating that *Pfiesteria* spp. have ichthyotoxic strains (*e.g.,* Moeller *et al.,* 2001), and that TOX-B *Pfiesteria* spp., separated from live fish for several weeks, can retain residual toxicity (Springer *et al.,* 2002). This study also showed that the same water-soluble toxin detected from SFBs with toxic *Pfiesteria* spp. in the previous studies was found in pure *P. shumwayae* or *P. piscicida* cultures grown with only nontoxic algal prey. Physical attack of fish by *Pfiesteria* helps weaken fish and may allow the toxin(s) to move into fish tissues more rapidly (Burkholder *et al.,* 2001c). *Pfiesteria* spp. may also be similar to certain other toxic dinoflagellates in having strains that differ in production of endotoxins and exotoxins. In ongoing research we are continuing to test these hypotheses with multiple strains.

Toxic *Pfiesteria* spp. strains are common in eutrophic estuaries, and maps of collection sites, collection methods, and detailed culturing procedures are available in the published literature. This study, considered with previously published information, shows the importance of testing multiple strains when assessing the potential for toxicity at the species or genus level, using appropriate techniques for culturing and toxin detection.

Acknowledgements

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Relating Cyanobacterial Abundance to Environmental Parameters in the Lower St. Johns River Estuary

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Abstract

The St. Johns River and estuary, a system located along the northeastern coast of Florida, has undergone extensive eutrophication, largely due to nutrient loading. The relationship between the occurrence and abundance of phytoplankton, including bloom-forming cyanobacteria, and water quality was characterized along the oligo- to mesohaline gradient in the Lower St. Johns River Basin (LSJRB) between November 2000 and July 2001. Absolute and relative cyanobacterial chlorophyll concentrations were determined using chemotaxonomic photopigments and matrix factorization techniques. The two oligohaline sites generally were more similar in terms of physicochemical parameters than the five mesohaline sites. A pronounced seasonal variation in phytoplankton abundance (as total chlorophyll *a*) occurred, with indications that conditions for biomass accumulation were most favorable during the summer months. Cyanobacterial chlorophyll *a* generally followed temporal and spatial trends with the greatest concentrations occurring at oligohaline sites, potentially suggesting the co-influence of nutrient loading and fresh water inflows upon phytoplankton assemblages.

Introduction

Phytoplankton biomass reflects the combined effects of hydrologic and water quality parameters and associated biological responses. The dynamic interplay of physical and chemical parameters creates gradients where species-specific responses enable bloom-forming taxa to establish large biomass accumulations. Although extensive knowledge exists concerning phytoplankton abundance, linkages between physical/ chemical factors and phytoplankton bloom initiation and proliferation within dynamic, estuarine waters remains poorly understood (Paerl, 1988). Even less is known about cyanobacterial blooms in tidally influenced black water systems like the St. Johns River.

Extensive cyanobacterial blooms occur annually throughout the St. Johns River Basin, a 300 mile-long system located in northeastern Florida, USA (Fig. 1). This annual accumulation of phytoplankton biomass has been attributed to significant point and non-point source nutrient loading. It has been associated with localized fish kills (from hypoxia/ anoxia), loss of submerged vegetation (from reduced water clarity and increased epiphyte biomass), wildlife mortalities, production of toxins (*i.e.,* microcystin, anatoxin, cylindrospermopsin), and human health issues. To clarify the ecological mechanisms underlying cyanobacterial bloom dynamics, the regulating factors for phytoplankton distribution with respect to the physical environment first must be identified. Here, we characterize cyanobacterial biomass along an oligo- to mesohaline gradient throughout the LSJRB (Fig. 1) during 2000 and 2001.

Materials and Methods

Physical, chemical, and phytoplankton samples were collected from subsurface and bottom waters on a seasonal basis from seven fixed sites (Fig. 1). Physical (temperature, salinity, pH, turbidity) and chemical (dissolved oxygen, inorganic and organic nitrogen, inorganic phosphate) parameters were measured using water quality multiprobes (YSI and HydroLab) and standard methods (Parsons *et al.,* 1984). Phytoplankton biomass (as chlorophyll *a*) was determined using chemotaxonomic photopigments derived using high-performance liquid chromatography (HPLC) (Millie *et al.,* 1993, 2002). Absolute and relative cyanobacterial contributions to chlorophyll *a* were derived using phylogenetic group-specific photopigments and CHEMical TAXonomy (CHEMTAX) matrix factorization. CHEMTAX estimates the contribution of a given phylogenetic group, defined in terms of the pigment complex, to chlorophyll *a* based on carotenoid: chlorophyll *a* ratios (Mackey, 1998).

A principle component analysis (PCA), utilizing Euclidean distances, characterized sampling sites with respect to physical parameters. A paired T-test assessed differences in total chlorophyll concentrations between subsurface and

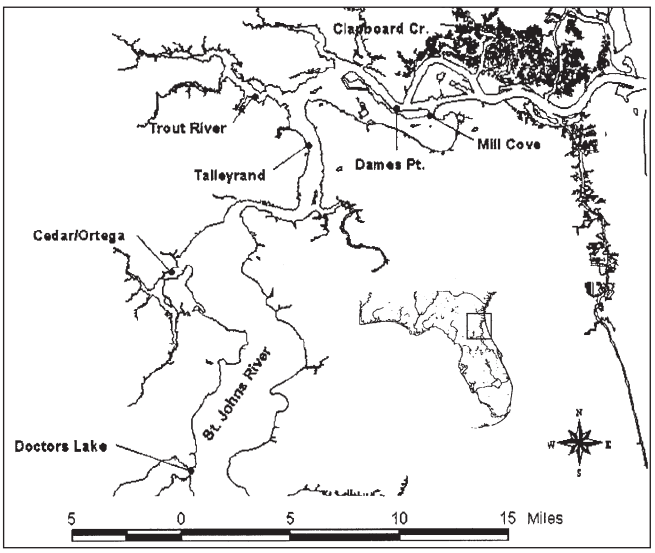

Figure 1 Location of sampling sites within the Lower St. Johns River Basin. Inset figure places study area relative to the state of Florida (USA).

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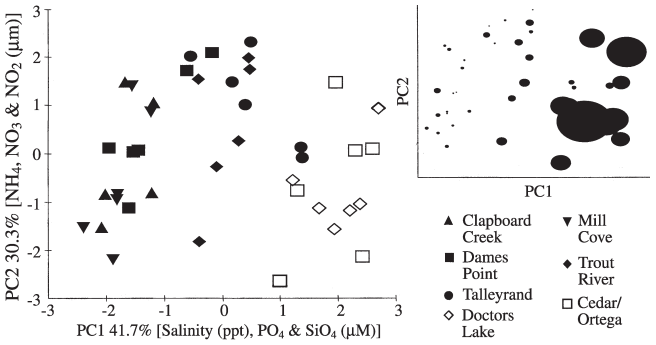

Figure 2 Two-dimensional principal component analysis ordination of replicate sampling days (5) for sites during July 2001 based on physical and chemical parameters. Inset figure depicts relative cyanobacterial chlorophyll *a* concentrations superimposed onto sample sites (the larger the bubble, the greater the concentration) to illustrate distributions.

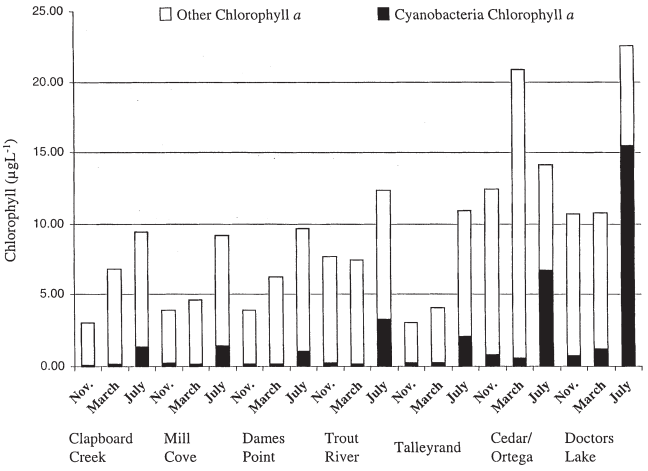

Figure 3 Cyanobacteria chlorophyll *a* and total chlorophyll *a* within subsurface samples.

bottom waters. A Pearson product moment correlation determined the relationship of absolute cyanobacteria with physical parameters.

Results and Discussion

The first and second components of the PCA explained ca. 72% of the total variability along sites during July 2001 and included physical and chemical parameters (salinity, biologically important nutrients) indicative of tidal flushing/freshwater inflows (Fig. 2). Analysis for the November/December 2000 and March/April 2001 sampling events produced relatively similar distributions. The oligohaline sites (Doctors Lake, Cedar/Ortega River) generally were more similar (in terms of chemical/physical parameters) than the five mesohaline sites (Clapboard Creek, Mill Cove, Dames Point, Talleyrand, Trout River).

Photopigments identified as biomarkers for phylogenetic groups within the LSJRB included: fucoxanthin (diatoms), alloxanthin (cryptophytes), neoxanthin, violaxanthin, lutein, chlorophyll *b* (chlorophytes), zeaxanthin (cyanobacteria), peridinin (dinoflagellates), and chlorophyll *a* (all algae). Chlorophyll *a* concentrations within subsurface and bottom waters were equivalent ($P > 0.05$), indicating a well-mixed water column.

Phytoplankton populations within the LSJRB were highly dynamic. A pronounced seasonal variation in phytoplankton abundance occurred, with indications that conditions for phytoplankton accumulation were most favorable during summer (also see Demort and Bowman, 1985, Aldridge *et al.,* 1998). The lowest chlorophyll *a* concentrations occurred in November/December, with concentrations increasing in mid-late spring (March/April) before obtaining the greatest values in March and July. Chlorophyll *a* concentrations (mean ± SE) across all sampling sites during November, March, and July were 6.05 ± 0.47, 8.71 ± 0.67, and 12.47 ± 1.97 µg L^{-1}, respectively. Total chlorophyll *a* concentrations increased with increasing distance from the estuary mouth, with the greatest concentrations (17.93 and 34.25 µg L^{-1}) observed at oligohaline sites (*i.e.,* Cedar/ Ortega, Doctors Lake; Fig. 3).

Phytoplankton composition was most diverse during March/April and July, with a dominance shift from diatoms to cyanobacteria during July at the oligohaline sites (Millie, unpubl. data, Demort and Bowman, 1985; Fig. 3). Absolute (and relative) cyanobacterial contribution to chlorophyll *a* varied spatially and temporally, ranging from 2.41 to 15.5 µg L^{-1} (4.73% to 70.9%). Similar to that observed for total chlorophyll *a*, the greatest cyanobacterial chlorophyll *a* concentrations (9.7 µg L^{-1} and 15.5 µg L^{-1}) occurred at the oligohaline sites. Absolute and relative cyanobacteria chlorophyll was inversely associated with salinity, particularly in March/April and July, but had no consistent association with temperature (Table 1, Fig. 4).

As part of the National Oceanic Atmospheric Admin-

Table 1 Pearson Product Moment Correlation of subsurface and bottom total phytoplankton/ cyanobacterial chlorophyll *a* concentration (µg L^{-1}) with depth-specific salinity and temperature.

Depth	Parameter	November–December	March–April	July
Subsurface	Salinity	–0.87[a] / –0.92[b]	–0.9[b] / –0.9[b]	–0.78[a] /–0.87[a]
	Temperature	–0.67 / –0.63	–0.1 / –0.34	0.82 / 0.63
Bottom	Salinity	–0.60 / –0.86[a]	–0.88[b] / 0.01	-0.88[b] /–0.96[c]
	Temperature	0.71 / 0.60	–0.57 / 0.32	–0.42 / –0.33

[a, b, c] indicates significance at 0.05, 0.01, and 0.001 probability levels, repectively.

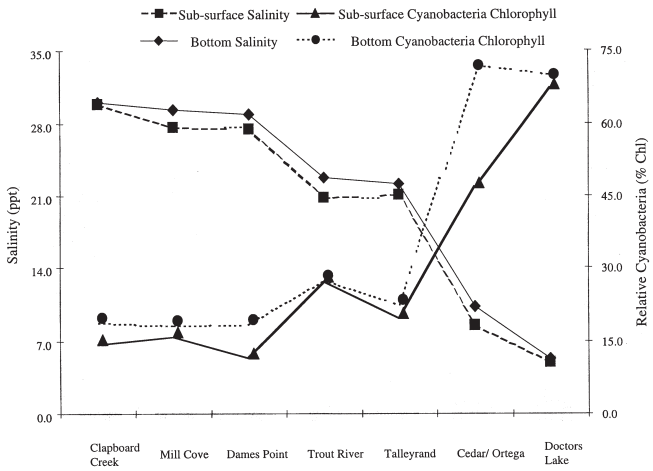

Figure 4 Cyanobacteria chlorophyll *a* and salinity values within subsurface and bottom waters during July 2001.

istration program "Monitoring and Event Response of Harmful Algal Blooms," this work provided baseline information concerning the distribution of phytoplankton accumulation within the LSJRB and the potential linkages of cyanobacteria with hydrologic alterations. It is critical to establish linkages between water quality prior to, during, and following any hydrologic modifications. Such water quality data then can be directed toward a comprehensive monitoring network, and generate positive feedback for determining the timing and extent of phytoplankton blooms in relation to water inflow and nutrient inputs.

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Composition and Distribution of *Pseudo-nitzschia* from Guanabara Bay, Brazil: The Role of Salinity, Based on Field and Culture Observations

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Abstract

The composition and distribution of *Pseudo-nitzschia* from Guanabara Bay, Brazil, a polluted estuary, was based on bottle and net samples collected weekly (Jul 1998–Dec 2000) at two contrasting sites: Urca (eutrophic, more saline) and Ramos (hypereutrophic, less saline). All 7 species identified (*P. brasiliana, P. cuspidata, P. delicatissima, P. fraudulenta, P. multistriata, P. pungens* and *P. pseudodelicatissima*-type) were found at both sites, but the genus was more frequent and with a higher number of species per sample at Urca. The "delicatissima complex" dominated at both sites, whereas the "seriata complex" occurred often at Urca and occasionally at Ramos. Total cell density (10^3–10^6 cells L^{-1}) was lower during the rainy season (summer), especially at Ramos. The effect of salinity was tested on cultures of *P. pungens* (BG8) and *P. multistriata* (BG6) isolated from the bay. Both species grew well in the 20–40 range when gradually acclimated, but the range was narrower (25–40) in a salinity shock experiment. Our results indicate that *Pseudo-nitzschia* species at Ramos are part of the resident populations rather than just being carried by tidal currents. Other factors that may control the occurrence, abundance and species richness at Ramos, especially in regard to the "seriata complex," need further investigation.

Introduction

The genus *Pseudo-nitzschia* is widely distributed in marine systems and is presented as being restricted to marine plankton (Hasle and Syvertsen, 1997). Indeed, cell abundance in field samples shows that the genus has a distinct preference for more saline waters. For instance, in Willapa Bay, USA, *Pseudo-nitzschia* spp. only became abundant after salinities were above 29 (Sayce and Horner, 1996). Lower salinity, however, does not seem to exclude *Pseudo-nitzschia* species, for example, as found in the Mississipi river estuary, USA, where they were detected over a salinity range of 0 to 36 (Dortch *et al.,* 1997). Salinity studies in the laboratory (*e.g.,* Reap, 1991; Jackson *et al.,* 1992; Lundholm *et al.,* 1997) have included colder water (temperate) isolates of *P. multiseries, P. pungens,* and *P. pseudodelicatissima.* The present study adds to this list by testing the effects of salinity on the relatively little studied *P. multistriata,* and on a tropical strain of *P. pungens,* both isolated from Guanabara Bay, Brazil. These are species that have tested positive for domoic acid in culture (Rhodes *et al.,* 1996; Sarno and Dahlmann, 2000), but have not yet been related to Amnesic Shellfish Poisoning. Data on distributional patterns from Guanabara Bay are also presented. Our goal is to combine field and culture data to provide stronger hypotheses for understanding *Pseudo-nitzschia* distributional patterns in nature.

Materials and Methods

The distribution of *Pseudo-nitzschia* at the genus level was based on bottle samples collected weekly (Jul 1998– Dec 2000) at the surface, at two contrasting sites: Urca (mesotrophic, more saline: 29–35) and Ramos (hypereutrophic, less saline: 17–30). *Pseudo-nitzschia* counts were done with the Utermöhl's settling technique. Species composition was determined from selected net (20 µm-mesh) samples that had been collected simultaneously with the bottle samples. Cells were cleaned of the organic material and prepared (Hasle and Fryxell, 1970) for study by light mi-

Table 1 Experimental conditions used with cultures of *P. pungens* (BG8) and *P. multistriata* (BG6).

Condition	Acclimatization Study	Shock Treatment
Acclimatization	Gradual, degree by degree; culture remained at each salinity for 1 week before following transfer	None
Salinities tested[1]	15, 20, 25, 30, 35 and 40[2]	0, 5, 10, 15, 20, 25, 30, 35, 40, 45
Culture before experiments	Kept in exponential growth for 6 days	Kept in exponential growth for two weeks at salinity 30 (control)
Volume of the culture/flask	100 mL in 250-mL flasks	50 mL in 100-mL flasks
Replicates	4	2–4
Sub-sampling scheme	At inoculation, day 1 and every 2nd day until stationary phase	At inoculation and after 96 hours (as done in ecotoxicology)

[1]The effect of salinity on *P. multistriata* was tested by the one-way analysis of variance and that on *P. pungens* by the non-parametric Kruskall-Wallis test (the assumption of equal variances was not verified for the latter); [2]not fully acclimated beyond 15 and 40.

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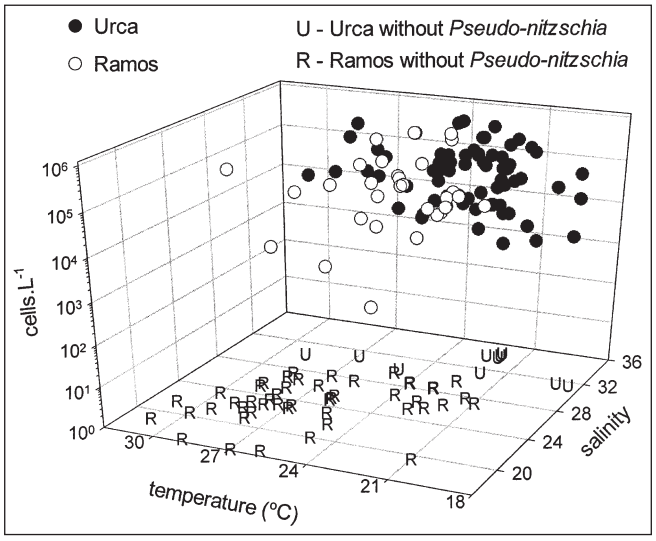

Figure 1 Distribution of the genus *Pseudo-nitzschia* in Guanabara Bay (site Urca and site Ramos), according to a salinity × temperature plot.

croscopy and scanning electron microscopy. The effect of salinity was tested on non-axenic batch cultures of *P. pungens* (BG8) and *P. multistriata* (BG6) isolated from Urca. Cultures of each species were routinely maintained in f/2 medium, salinity 30, 22–23°C, and 12:12 h light:dark cycle at 138–147 µmol m^{-2} s^{-1}. Two types of experiments were carried out (Table 1) to test the effect of salinity on population growth, which was evaluated by cell counts. Salinity from field samples was measured by silver nitrate titration and from culture samples with an Atago N-50E hand refractometer.

Results and Discussion

The genus *Pseudo-nitzschia* was found at both sites, but it was more frequent at Urca than at Ramos (in 84% and 38% of the samples, respectively). Total cell density (10^3–10^6 cells L^{-1}) was lower during the rainy season (austral summer), especially at Ramos where *Pseudo-nitzschia* were not detected during periods of higher water temperature and lower salinity (Fig. 1). The 7 species identified (*P. brasiliana, P. cuspidata, P. delicatissima, P. fraudulenta, P. multistriata, P. pungens* and *P. pseudodelicatissima*-type) were found at both sites, but with a higher number of species per sample at Urca (data not shown). Species of the "delicatissima complex" dominated at both sites (Fig. 2), whereas those of the "seriata complex," with common occurrences at Urca, were found at Ramos on few occasions and only in concentrated net samples (data not shown).

P. pseudodelicatissima-type may have included *P. pseudodelicatissima, P. calliantha* and/or *P. caciantha* (see Lundholm *et al.,* 2003).

In field samples, *P. pungens* and *P. multistriata* were found in salinities as low as 23. In culture, both species grew best in the 20/25–40 range when gradually acclimated, but the range was narrower (25/30–40) in the salinity shock experiment, especially for *P. multistriata* that did not grow at 15 and below, even when acclimated (Fig. 3). These 2 types of experiments tested different scenarios for Guanabara Bay. The acclimatization study simulated the conditions that could allow resident populations to develop at lower salinities. The shock treatment simulated a situation in which the populations are not autoctonous, that is, they would be carried into the bay by tidal currents and subjected to lower salinity due to sudden and intense wind-driven turbulence. The comparison of field and culture data indicates that *Pseudo-nitzschia* species at Ramos can be part of the resident population.

Our data demonstrate that salinity is one of the factors that may control the abundance and composition of *Pseudo-nitzschia* species in Guanabara Bay. The comparison of the species composition between Urca and Ramos ("*delicatissima* complex" vs. "*seriata* complex"), as well as between the growth of *P. pungens* and *P. multistriata* in culture, raises an interesting question for future studies. Although growth rate was similar, under our experimental conditions with batch cultures, the growth curve was different: *P. multistriata* was not able to maintain biomass for longer than 2–4 days during the plateau phase, whereas *P. pungens* was able to remain viable during the plateau for at least 10 days (data not shown). Our hypothesis for future research

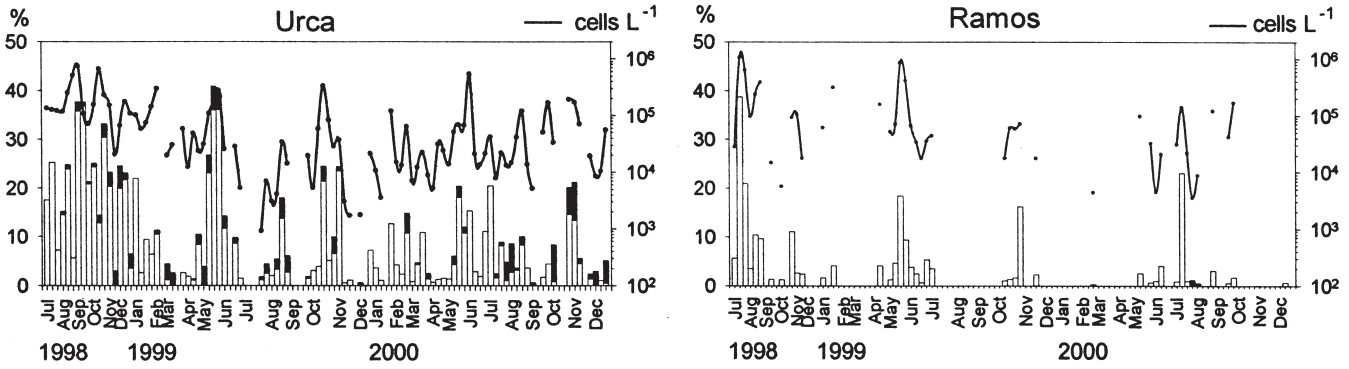

Figure 2 Distribution of total cell density (cells L^{-1}) of *Pseudo-nitzschia* and percentages of the "*delicatissima* complex" (width < 3 µm) and of the "*seriata* complex" (width ≥ 3 µm) to total cell density of the microphytoplankton fraction (cells > 20 µm of largest measurement).

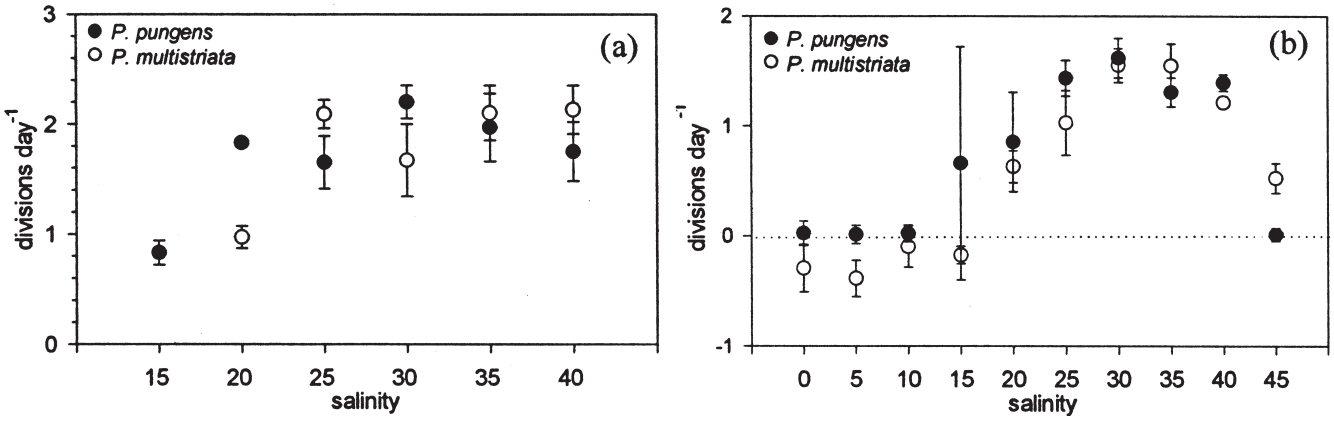

Figure 3 Growth rates of *P. pungens* and *P. multistriata.* (**a**) In the acclimatization experiment, *P. pungens* showed no significant difference for 20–40 ($P < 0.01$) and *P. multistriata* for 25–40 ($P < 0.01$). (**b**) In the shock treatment, *P. pungens* showed no significant difference for 25–40 ($P < 0.01$) and *P. multistriata* for 30–40 ($P < 0.01$).

is that the effects of salinity are nutrient-dependent. The application of this hypothesis to field data is that it is possible that different degrees of eutrophication found in Guanabara Bay waters can play a role in *Pseudo-nitzschia* distribution at the species level.

Acknowledgements

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A Comparison of Photosynthetic and Heterotrophic Carbon Acquisition by *Aureococcus anophagefferens*

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Abstract

Blooms of *Aureococcus anophagefferens* occur in coastal bays along the east coast of the United States during the spring and early summer. These so-called "brown tides" occur when inorganic nutrients are depleted relative to organic nutrients, and it has been observed that *A. anophagefferens* can take up and grow on dissolved organic nutrients, particularly dissolved organic N (DON). It has been suggested that dissolved organic matter (DOM) also provides C for growth and that this may substantially subsidize photosynthetic C uptake. In order to quantify this potential subsidy, we compared uptake rates of selected organic C and N substrates with rates of photosynthetic C acquisition and rates of peptide hydrolysis in natural populations collected from two sites in Chincoteague Bay: one that has experienced seasonal brown tide blooms in each of the last 4 years and one that has not. Sampling took place between March and June 2002. A brown tide bloom developed at one site at the end of May and persisted through mid-June. Results suggest that during the day, photosynthetic C fixation is the dominant mode of C acquisition but that it is insufficient for meeting the total cellular C demand. During the brown tide bloom, N uptake was dominated by urea, but urea C did not contribute substantially to growth. Other organic compounds tested did not contribute much to N or C uptake, and peptide hydrolysis rates were lower than those measured during a brown tide bloom on Long Island, suggesting this was not an important pathway for mobilizing DOM in this system.

Introduction

Blooms of the brown tide pelagophyte, *Aureococcus anophagefferens,* occur seasonally along the eastern seaboard of the USA, where high concentrations of dissolved organic nitrogen (DON) relative to dissolved inorganic N (DIN) are thought to fuel their growth (Berg *et al.,* 1997, Gobler and Sañudo-Wilhelmy 2001, Glibert *et al.,* 2001). In addition to providing N, dissolved organic matter (DOM) can also provide carbon to cells. Previous work has illustrated that *A. anophagefferens* can take up organic C compounds (Dzurica *et al.,* 1989, Mulholland *et al.,* 2002). This may be advantageous during blooms when cell densities are high (*e.g.,* 10^6 cells mL^{-1}) and self-shading becomes significant. Despite the fact that blooms of brown tide are increasing in frequency and geographical extent, little is known about how the dominant pathway of C acquisition by this species promotes growth and the development of blooms. There are a variety of organic compounds available to cells in nature. For example, uptake of urea and amino acids has been observed in a number of phytoplankton taxa (*i.e.,* Antia *et al.,* 1991), including *A. anophagefferens* (Berg *et al.,* 1997, Mulholland *et al.,* 2002), and the contribution of these compounds to the N nutrition has been examined in a number of systems. However, contribution of these compounds to the C nutrition of *A. anophagefferens* has not been examined. In addition to C uptake itself, there is also the question of organic matter size. A variety of larger labile organic compounds such as proteins and peptides may be unavailable to marine microbes because they are too large for transport into the cell. We therefore examined the capacity of *A. anophagefferens* both to take up organic C compounds and to degrade large organic compounds extracellularly.

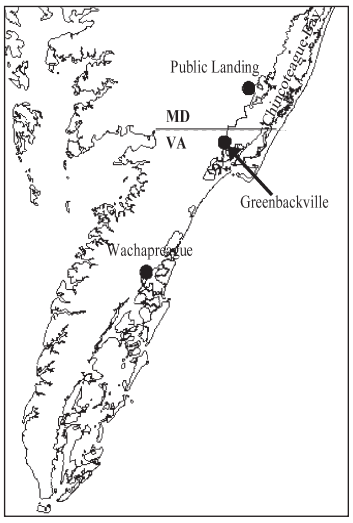

Figure 1 Map of the study site.

Since 1999, *A. anophagefferens* cell densities have increased and a number of blooms have been reported in Chincoteague Bay, MD. In order to determine how nutrient interactions affect bloom formation, we compared nutrient concentrations, rates of photosynthesis and uptake of organic and inorganic N and organic C at two sites in Chincoteague Bay: one at Public Landing, MD (PL), where blooms have been reported, and one in Greenbackville, VA (GB), where blooms have not been previously reported.

Materials and Methods

Water samples were collected from sites in Chincoteague Bay in acid-cleaned carboys and transported to a field laboratory at Wallops Island, VA (Fig. 1). Within 30 minutes of collection, nutrient samples were filtered and frozen in acid-cleaned bottles. Dissolved nutrients were measured colorimetrically (Parsons *et al.,* 1984) and dissolved free amino acids (DFAA) were measured using high performance liquid chromatography (HPLC) (Lindroth and Mopper 1979) at Old Dominion University. Incubations for rate process measurements were initiated within 1 hour of sample collection. Whole water and <5.0 μm filtered samples were placed in acid-cleaned polycarbonate bottles and initiated by adding the appropriate labeled substrate. Uptake

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Table 1 Salinity, temperature, chlorophyll *a*, *Aureococcus anophagefferens* abundance and nutrients at 2 sites in Chincoteague Bay, MD and VA.

Date	Salinity	Temperature °C	Chl *a* (μg chl l^{-1})	*A. a.* abundance (cells ml^{-1} x 10^3)	PC:PN Molar Ratio	NH_4^+ (μM)	NO_3^- (μM)	Urea (μM)	DFAA (μM)	DOC (μM)
Greenbackville, VA:										
03/01/02	33.3	6.5	0.75 (0.04)	0.027	9.1	0.20	0.01	0.00	0.17	215
04/05/02	30.4	12.9	1.15 (0.05)	0	8.8	0.88	0.21	0.26	0.46	277
05/02/02	30.8	17.5	8.7 (0.42)	0	11.3	0.62	0.13	2.57	0.33	270
05/15/02	31.6	17.5	1.70 (0.23)	0.018	17.5	0.58	0.21	2.04	0.53	245
05/30/02	32	25.7	3.53 (1.5)	0.093	10.7	0.05	0.01	1.49	0.28	276
06/06/02			7.38 (0.75)	0.096						258
06/12/02	33.7	24.3	5.09 (2.4)	0.075	9.4	1.17	0.30	2.87	0.52	229
Public Landing, MD:										
03/01/02	33.2	6.2	1.22 (0.24)	0.017 (0.006)	8.1	0.07	0.02	0.00	0.20	366
04/05/02	30.5	13.6	1.83 (0.38)	0.009	8.9	0.50	0.01	1.22	0.39	360
05/02/02	30	17.7	5.23 (0.19)	15.2 (1.6)	9.8	0.50	0.01	3.40	0.52	381
05/15/02	30.6	18.9	2.37 (0.16)	0.44	9.3	0.23	0.01	2.08	0.25	369
05/30/02	30.5	27.5	10.2 (1.1)	396 (16)	12.3	0.13	0.01	2.40	0.60	429
06/06/02			14.4 (1.5)	588 (51)	11.8	0.05		2.80	0.30	424
06/12/02	32.6	24.8	19.5 (1.5)	1139 (164)	14.0	0.05	0.01	4.52	0.35	491

rates for NH_4^+, NO_3^-, urea, amino acids, HCO_3^-, and glucose were measured using highly enriched (>98%) stable isotopes (^{15}N and ^{13}C) (Glibert and Capone 1993; Mulholland *et al.,* 2002). Incubations were initiated at mid-day with the addition of tracer and terminated after <20 minutes (except $H^{13}CO_3^-$ incubations, which were 1 hour) by gentle filtration through precombusted GF/F (nominal pore size of 0.8 mm) or silver (0.2 μm) filters. Photosynthetic uptake of C was also measured using the ^{14}C method (Parsons *et al.,* 1984). Peptide hydrolysis was measured using Lucifer Yellow Anhydride (LYA)-labeled tetraalanine (Pantoja *et al.,* 1997, Mulholland *et al.,* 2002).

Results and Discussion

Urea was the most abundant dissolved nitrogenous nutrient at both sites during May and June (Table 1). DFAA and NH_4^+ concentrations were measurable throughout the study period but dissolved NO_3^- concentrations were always low or at the limit of analytical detection. Chlorophyll *a* biomass increased during May at both sites but was twice as high at PL, where a brown tide bloom (1.1 million cells mL^{-1}) occurred during late May and June, than at GB. Dissolved organic carbon (DOC) concentrations were higher at PL than at GB throughout the study period.

Urea was the dominant form of N taken up onto GF/F filters during May and June at both sites in Chincoteague Bay (Fig. 2). During the brown tide bloom at PL, total N uptake was much higher than at GB. The bulk of the N uptake at PL was accomplished by the <5.0 μm size fraction which included *A. anophagefferens.* At GB, most of the N uptake was by cells larger than 5.0 μm (data not shown).

Photosynthetic uptake of HCO_3^- provided the bulk of the measured C uptake onto GF/F filters for the whole water (data not shown) and the <5.0 μm size fraction (Fig. 3) during this study. Total measured C uptake from photosynthesis and selected organic compounds was insufficient to meet the cellular C demand estimated from hourly N uptake rates and molar C:N ratios of particulate material during the brown tide bloom at PL (Table 2). If we assume that photosynthetic C uptake is confined to daylight hours while N uptake from NH_4^+ and urea, but not NO_3^-, proceeds dur-

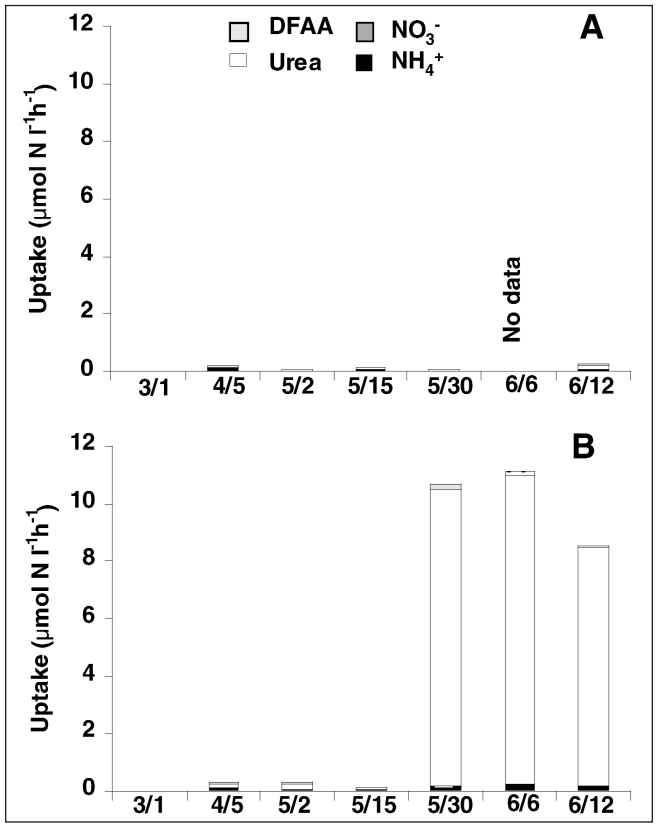

Figure 2 N uptake in <5.0 μm water samples at **A** Greenbackville, VA, and **B** Public Landing, MD.

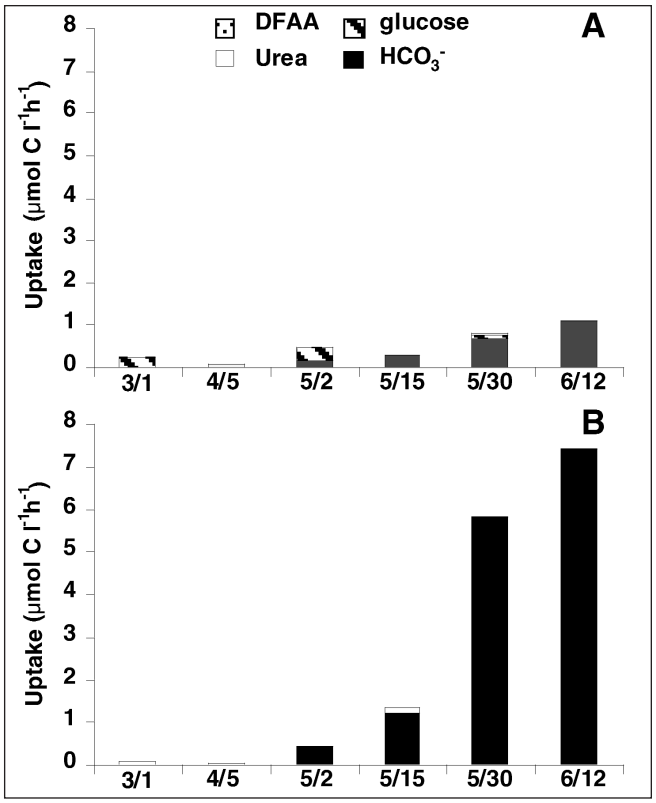

Figure 3 C uptake in water <5.0 µm collected on GF/F filters (0.8 mm) from **A** Greenbackville, VA, and **B** Public Landing, MD.

ing the day and night, the deficit in total C uptake relative to total N uptake is even more pronounced. Incubations were conducted mid-day to approximate maximum photosynthetic rates, however, because photosynthetic rates vary throughout the daylight hours, it is possible that at least a portion of the imbalance was due to underestimates of daily photosynthetic rates. Investigations are also underway to assess dark uptake of the organic C substrates used in this study and to identify other organic compounds in the environment that may be providing additional C for cell growth. When we used 0.2 µm filters in our uptake studies, rates of organic C uptake were high, suggesting that bacteria were using organic C substrates during the day and possibly competing with *A. anophagefferens* for the compounds tested (Fig. 4).

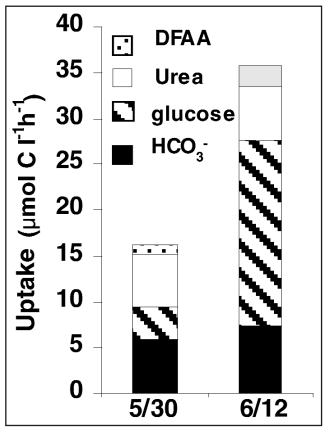

Figure 4 C uptake in water <5.0 mm collected on 0.2 µm filters.

Rate constants for peptide hydrolysis were low (~1 d^{-1}, data not shown) compared with those observed during a brown tide bloom on Long Island, NY in 2000 (~7 d^{-1}) (Mulholland *et al.,* 2002). Rates of peptide hydrolysis may be inhibited by the presence of other labile N and C sources such as urea.

Table 2 Ratio of the total C:total N uptake and the molar PC:PN ratio during May and June 2002.

	Date	Total C:N Uptake	PC:PN Molar Ratio
Greenbackville, VA	5/2	6.5	14.6
<5.0 gm size fraction	5/30	9.9	12.9
	6/12	4.3	11.3
	5/2	14.7	11.3
whole water	5/30	0.6	10.7
	6/12	10.6	9.4
Public Landing, MD	5/2	1.6	11.4
< 5.0 µm size fraction	5/30	0.56	12.9
	6/12	0.87	14.1
	5/2	0.69	9.8
whole water	5/30	0.69	12.3
	6/12	1.9	14.0

Because urea was abundant, it, rather than amino acids, was the primary source of organic N at PL during the bloom. Urea concentrations were similar at GB and PL, but at GB, inorganic N concentrations were never depleted and no bloom occurred. Dissolved organic matter can be an important source of N and C for *A. anophagefferens* (Mulholland *et al.,* 2002) but, because the source and type of organic material can vary among sites, estimates of organic C uptake may vary. Bacterial processes may complicate the picture since these organisms may compete with *A. anophagefferens* for organic material.

Acknowledgements

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Occurrence and Ecology of the Dinoflagellate *Karlodinium micrum* in Estuaries of North Carolina, USA

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Abstract

The dinoflagellate *Karlodinium micrum* (Leadbeater et Dodge) J. Larsen (=*Gyrodinium galatheanum* (Braarud) Taylor) was documented in estuaries in North Carolina since 1998. It is possible that *K. micrum* was present in North Carolina prior to this time but was identified as *Pfiesteria piscicida*. The NC Division of Water Quality has conducted an extensive estuarine phytoplankton monitoring program for several years, and *K. micrum* was routinely found during regular monitoring as well as during fish kill events. *Karlodinium micrum* was present across wide ranges of temperature, salinity, and nutrient concentrations and was most common during June–August. This dinoflagellate rarely exceeded 5,000 cells/mL and only twice exceeded 30,000 cells/mL. Continued monitoring for this species is needed to learn more about its ecological role and potential for fish kills.

Introduction

The estuarine dinoflagellate *Karlodinium micrum* is found in brackish systems along the US Atlantic Coast, southwest Africa, and the North Sea (Nielsen, 1993; Li *et al.,* 2000, Kempton *et al.,* 2002; Marshall *et al.,* 2000). This species is reported to cause fish kills in estuaries at cell densities of 30,000 cells/mL or higher (Nielsen, 1993; A. Place, University of Maryland, USA, pers. comm.). In the laboratory, fish kills can be induced by *K. micrum* concentrations of 5,000–10,000 cells/mL (Deeds *et al.,* 2002; A. Place, pers. comm.). On the US Atlantic Coast, *K. micrum* has been responsible for fish kills in brackish ponds (Deeds *et al.,* 2002; Kempton *et al.,* 2002). Little is known about the ecology of *K. micrum* in estuarine river systems, so data collected from coastal rivers in North Carolina are examined here.

Materials and Methods

Environmental data and samples for phytoplankton and nutrients were collected by the North Carolina Division of Water Quality (NC DWQ) during 1999–2001 (Table 1). Sampling runs were conducted monthly during February, June to September, and November in the Pamlico and lower Neuse rivers and monthly from April to December in the coastal New River (Fig. 1. Due to space constraints, only the Pamlico and New Rivers are shown here. There were three *Karlodinium micrum* blooms with cell densities >5,000 cells/mL recorded in the Neuse River during 1999–2001 (Northwest Creek, 1 Sept. 2000, 5,800 cells/mL; Broad Creek, 3 Aug. 2001, 5,400 cells/mL; Broad Creek, 22 Aug. 2001, 5,400 cells/mL). Additional sampling was conducted by "rapid response teams" on the Pamlico and lower Neuse Rivers. These teams monitored their respective rivers more frequently and collected samples associated with algal blooms and fish kills throughout the year. Water samples were collected for phytoplankton and nutrients (total nitrogen (TN = NO_2+NO_3 + total Kjeldahl nitrogen), total phosphorous (TP)). Subsurface measurements of dissolved oxygen (DO), pH, salinity, and temperature were collected using a Hydrolab (model Surveyor 4, Hydrolab Corp., Austin, TX, USA). Phytoplankton samples were preserved with acidic Lugol's solution (modified from Vollenweider, 1974 (glycerin added to preserve flagellae)) in the field and shipped on ice (approximately 4°C) within 24–72 hours to the laboratory. Samples were settled in Utermohl counting chambers and analyzed at 500× using a Leitz Diavert inverted microscope.

It was not until September 1998 that DWQ phycologists could discern between *Pfiesteria* and *Karlodinium micrum.* Since *K. micrum* bears a close physical resemblance to

Table 1 Data ranges according to *Karlodinium micrum* cell concentrations in the Neuse, Pamlico, and New River estuaries of North Carolina, USA.

	Temperature (°C)	pH	Salinity (ppt)	Total Nitrogen (mg/L)	Total Phosphorous (mg/L)
All *K. micrum* samples (20–32,200 cells/mL; n = 309)	2 to 33 (n = 283)	6 to 9 (n = 286)	0 to 30 (n = 287)	0.01 to 6.3 (n = 242)	0.01 to 0.51 (n = 242)
Samples with >5,000 cells/mL (n = 21)	14 to 31 (n = 21)	7 to 9 (n = 21)	3 to 22 (n = 21)	0.2 to 3.6 (n 14)	0.03 to 0.23 (n = 14)
Samples with >30,000 cells/mL (n = 2)	15 to 26 (n = 2)	7 to 8 (n = 2)	7 to 10 (n = 2)	1.1 (n = 1)	0 (n = 1)

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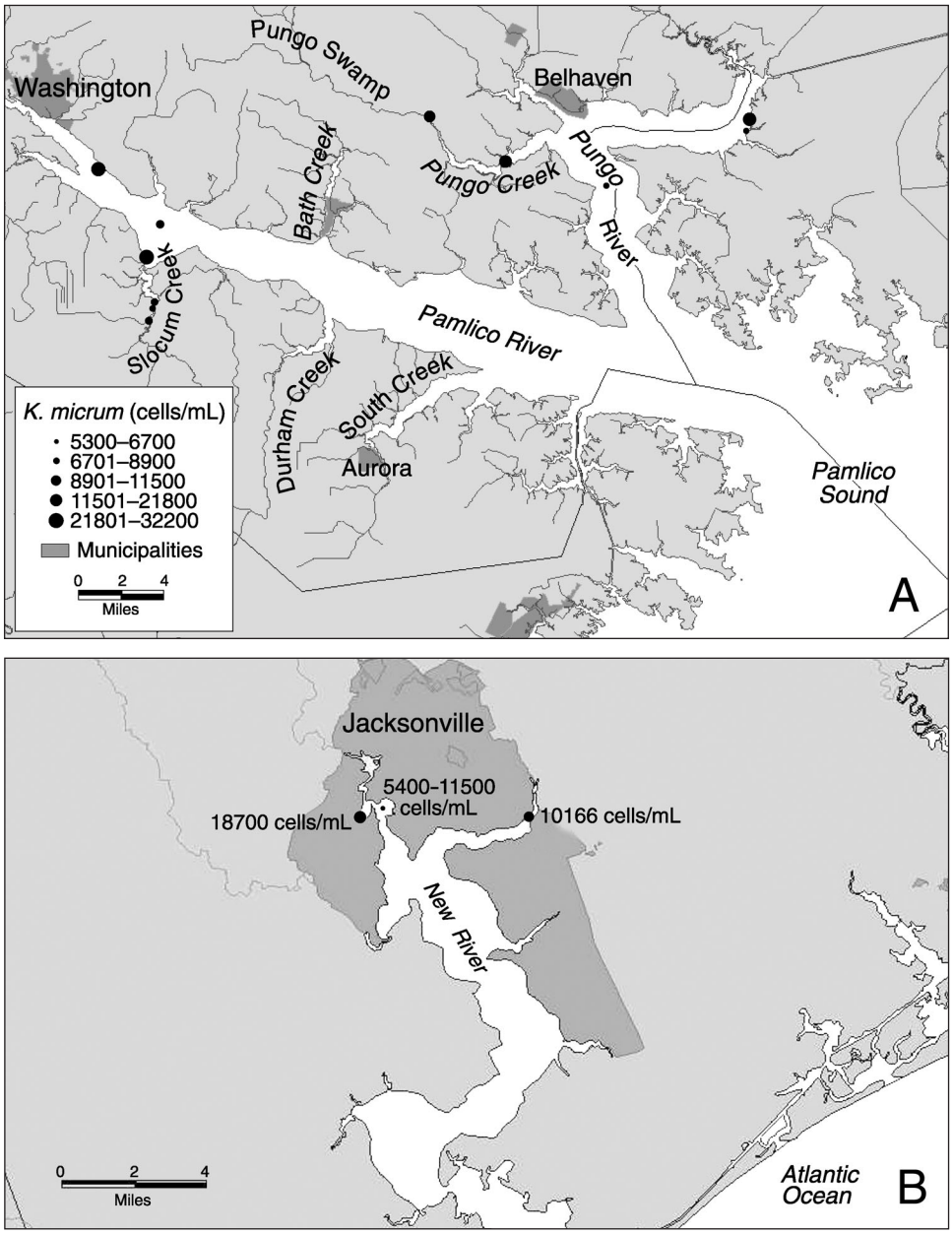

Figure 1 Concentrations of *Karlodinium micrum* >5,000 cells/mL in the Pamlico (**A**) and New (**B**) rivers in North Carolina, USA.

Pfiesteria, it is considered to be a "*Pfiesteria*-like" dinoflagellate (Burkholder *et al.,* 2001). *Pfiesteria piscicida* and *Pfiesteria shumwayae* can only be definitively identified using scanning electron microscopy (Burkholder *et al.,* 2001), but the chloroplast arrangement in *K. micrum* (see Braarud, 1957; Daugbjerg *et al.,* 2000) can be used to informally differentiate *K. micrum* from *Pfiesteria* under light microscopy. It is possible that *K. micrum* was present in North Carolina before 1998 but was identified as *P. piscicida*.

Results and Discussion

Karlodinium micrum was found throughout the study in all three rivers at cell densities ranging 20–32,200 cells/mL. It was most often found during June–August and was associated with wide ranges of temperature, salinity, and nutrients. The environmental pH range was narrower (Table 1).

Despite its frequency, *Karlodinium micrum* was rarely found at the high concentrations associated with fish kills reported in the literature. It exceeded 5,000 cells/mL in only 7% of the samples (Table 1). The ranges of the physical and chemical parameters associated with these higher density samples were narrower but may reflect the smaller sample size (Table 1). Only two *Karlodinium micrum* blooms exceeded 30,000 cells/mL (Table 1). Both of these blooms occurred during fish kills in bays on the Pamlico River (Fig. 1). Environmental data collected during both events

Table 2 Data ranges reported for recent *Karlodinium micrum* studies.

	Location	Temperature (°C)	pH	Salinity (ppt)
Fensin (this study)	North Carolina, USA	14 to 31	7 to 9	3 to 22
Neilsen (1996)	Laboratory (Norway)	20 to 24	not reported	24
Li *et al.* (2000)	Chesapeake Bay, USA	6 to 28	not reported	7 to 18
Deeds *et al.* (2002)	Aquaculture facility on Chesapeake Bay	28 to 30	not reported	5 to 18
Kempton *et al.* (2002)	Water retention pond in South Carolina, USA	11 to 12	7	11

were similar to data collected during the other >5,000 cells/mL *K. micrum* sightings. One >30,000 cells/mL bloom occurred during summer and the other during winter (Blounts Bay, 30 Jun. 1999, 32,200 cells/mL, 11,000 fish; Chocowinity Bay, 11 Dec. 2001, 30,900 cells/mL, 80 fish). Water samples collected during the fish kill at Blounts Bay were examined with a standardized fish bioassay used to assess for potential toxicity of *Pfiesteria* and *Pfiesteria*-like dinoflagellates (North Carolina State University's Center for Applied Aquatic Ecology (NCSU-CAAE), Raleigh, NC, USA, Burkholder *et al.,* 2001). Fish collected from the kill were examined for pathogens (NCSU-CAAE and NCSU College of Veterinary Medicine (NCSU-CMV)). The bioassays were negative for fish disease and mortality, and no pathogens were found with the fish collected at the kill site (NCSU-CAAE and NSCU-VMC, pers. comms.). Fish bioassay and pathology studies were not conducted in conjunction with the winter fish kill at Chocowinity Bay.

Temperature, pH, and salinity field data collected during this study were comparable to data collected during recent *Karlodinium micrum* studies reported in the literature (Table 2). The data recorded for the majority of the >5,000 cells/mL samples in this study fall within the range of field data recorded for the Chesapeake Bay (Li *et al.,* 2000) and during the pond fish kills (Deeds *et al.,* 2002; Kempton *et al.,* 2002). The data recorded for the >5,000 cells/mL samples in this study encompass the temperature and are close to the salinity range for optimum growth of *K. micrum* found by Nielsen (1996). Since *Karlodinium micrum* in North Carolina is capable of exceeding the potentially ichthyotoxic cell concentration of 30,000 cells/mL, continued monitoring is necessary to find out more about its ecological role in US Mid-Atlantic estuaries.

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Geographic Trends in *Alexandrium* spp. Growth and Toxicity as a Function of Environmental Conditions

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Abstract

Dinoflagellates of the genus *Alexandrium* are found in marine environments around the world and are known to produce the neurotoxins responsible for the human illness paralytic shellfish poisoning (PSP). Bloom dynamics and *Alexandrium* physiology differ geographically. Trends in toxicity have been observed, with potency increasing for isolates from northern locations. Although extensive research has investigated the causes of *Alexandrium* toxicity, there is no consensus as to whether environmental factors play a direct role. The purpose of this project was to determine the effects of environmental conditions on growth and toxicity of geographically distinct *Alexandrium* isolates. Four isolates from waters off the northeastern coast of North America were grown under a range of temperature, irradiance, and salinity treatments in the laboratory. Growth rate and toxicity varied among isolates and in response to environmental conditions. Toxicity, however, was not a function of growth rate. The same geographic trend in toxicity previously reported was observed. Results suggest that the observed geographical differences in *Alexandrium* toxicity are, at least in part, driven directly by ambient temperature, irradiance, and salinity.

Introduction

The most common toxic dinoflagellates in waters off the northeast coast of North America are the PSP-causative organisms *Alexandrium tamarense* and *A. fundyense* species, containing a suite of saxitoxin congeners that act as sodium channel blockers. Geographic trends in toxicity have been observed for *Alexandrium* in this region (Maranda *et al.,* 1985; Anderson *et al.,* 1994). Higher toxicity was detected in northern isolates, the result of having higher proportions of more potent toxin congeners than the southern isolates. The purpose of this project was to determine the effects of temperature, irradiance, and salinity on the growth and toxicity of geographically distinct *Alexandrium* isolates in order to explain biogeographical differences in bloom dynamics and toxicity. Previous studies on *Alexandrium* growth in response to environmental factors exist (*e.g.,* Prakash *et al.,* 1971; White, 1978; Hall, 1982; Ogata *et al.,* 1987; Parkhill and Cembella, 1999; John and Flynn, 2000), and toxicity has been shown to vary with salinity (White, 1978), temperature (Hall, 1982; Ogata *et al.,* 1987), light (Ogata *et al.,* 1987), and nutrients (Hall, 1982; Boyer *et al.,* 1987; John and Flynn, 2000). Yet it remains debatable whether toxicity varies in direct response to environmental conditions and if there are isolate-specific responses.

Materials and Methods

Alexandrium isolates (Table 1) were grown and acclimated as described in Etheridge (2002). Temperature, growth irradiance, and salinity were varied independently, holding all other parameters at standard conditions. Experimental treatments consisted of 5 temperature (5–25°C), 6 irradiance (6–425 mmol photons m^{-2} s^{-1}), and 5 salinity (15–35 PSU) levels. After acclimation, cultures were harvested in mid-exponential phase for analysis. Given the scope of the isolates examined and experimental conditions, the experiments were not replicated. Growth phase and growth rates were determined based on a time course of *in vivo* fluorescence, cells were enumerated using an Olympus CH30 light microscope and a Sedgewick-Rafter counting chamber, and toxin analysis was performed using high performance liquid chromatography (HPLC; *e.g.,* Anderson *et al.,* 1994) as described in Etheridge (2002).

Results

Growth rates varied among isolates and in response to environmental factors (Fig. 1a–c). The highest growth rate observed was 0.68 d^{-1} for one of the isolates at high irradiance; however, average growth rates were approximately 0.3 d^{-1}. There were two general growth responses to ranges in temperature (Fig. 1a). Isolates from the St. Lawrence Estuary (StL), Bay of Fundy (BoF), and off Monhegan Island (MI) displayed optimal growth rates at 15°C. The isolate from Long Island Sound, Connecticut (CT) exhibited increased growth rates as temperature increased to 25°C.

Table 1 Species and geographic location of *Alexandrium* isolates.

Species	Isolate ID	Isolation Location
A. tamarense	StL	St. Lawrence Estuary, Canada (48°35N, 68°11W)
A. fundyense	BoF	Bay of Fundy, Maine (44°42N, 66°31W)
A. fundyense	MI	off Monhegan Island, Maine (43°34N, 69°27W)
A. tamarense	CT	Mumford Cove, Connecticut (41°20N, 72°01W)

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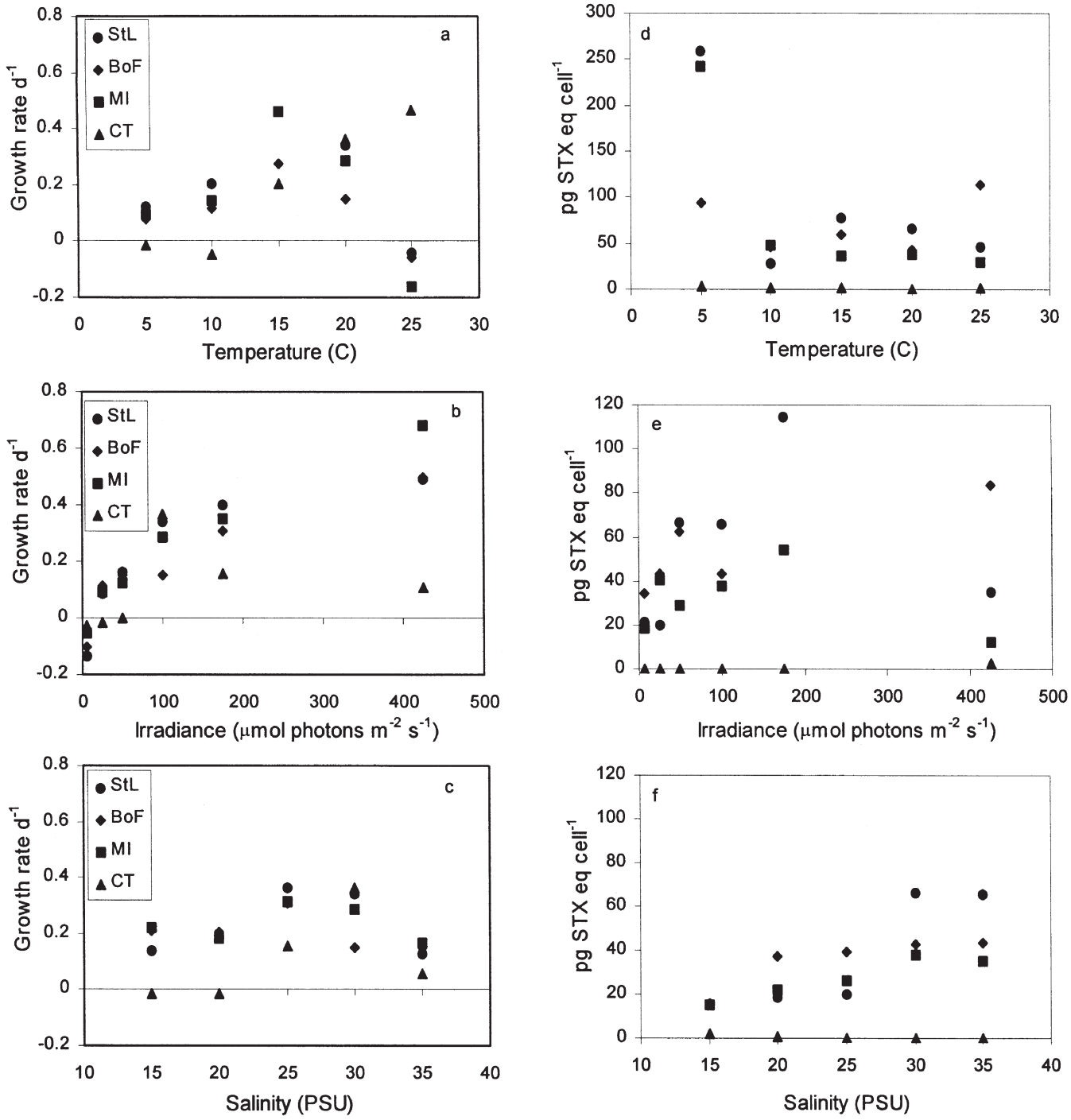

Figure 1 **a–c** Growth rates (d^{-1}) and **d–e** toxicity (pg STX eq $cell^{-1}$) for *Alexandrium* isolates (see legend) exposed to ranges in temperature, irradiance, and salinity.

Negative growth was observed for the CT isolate at 5°C and for isolates StL, BoF, and MI at 25°C. The net loss at 25°C coincided with observations of those isolates forming temporary cysts. There were also two different growth rate responses to ranges in irradiance (Fig. 1b). Isolates StL, BoF, and MI exhibited increased growth rates as irradiance increased, whereas the CT isolate displayed highest growth rates at an optimal irradiance of 100 µmol photons m^{-2} s^{-1}. Negative growth was observed for the CT isolate exposed to ≤25 mmol photons m^{-2} s^{-1} and for all isolates exposed to ≤6 mmol photons m^{-2} s^{-1}. Growth rates were less variable in response to salinity (Fig. 1c). The optimal salinity for isolates StL, BoF, and MI was 25 PSU, but 30 PSU for the CT isolate. Although isolates grew at 15 PSU, microscopic observations demonstrated changes in size and morphology when exposed to this low salinity. Cells were smaller by nearly 50% and cell surfaces were rough and convoluted, suggesting the cells were stressed.

Overall toxicity varied among isolates and in response to environmental conditions (Fig. 1d–f). On average, highest toxicity was observed in the StL, BoF, and MI isolates. All isolates had higher toxicity at the low temperature (Fig. 1d). For most isolates, the increase in toxicity when the temperature decreased from 10°C to 5°C was sharp; however, the CT isolate displayed a more gradual increase in toxicity with a decrease in temperature. The BoF isolate exhibited a notable increase in toxicity at 25°C. The StL, BoF, and MI isolates increased in toxicity with an increase in irradiance up to a critical irradiance, above which toxicity decreased (Fig. 1e). There was no difference in toxicity for the CT isolate at the low irradiances, yet there was an increase in cell toxicity at 425 µmol photons m^{-2} s^{-1} (Fig. 1e). Cell toxicity increased when exposed to increasing salinity (from 15 to 30 PSU) for isolates StL, BoF, and MI (Fig. 1f), whereas cell toxicity decreased when the CT isolate was exposed to increasing salinity (Fig. 1f).

Discussion

Results demonstrate that there is variability in growth rate and toxicity between isolates and in response to temperature, irradiance, and salinity. Thus, discrepancies in previous studies of environmental effects on *Alexandrium* may be the result of isolate-specific differences. In this study, temperature and irradiance had the greatest impact on *Alexandrium* growth and toxicity. Geographic trends were most evident with regard to cellular toxicity. The StL, BoF, and MI isolates were the most toxic, similar to previous observations (Anderson *et al.,* 1994). The most toxic isolates were from relatively similar environments, and they displayed similar responses to environmental conditions.

This research provides isolate-specific *Alexandrium* responses in growth and toxicity to a range of temperature, irradiance, and salinity conditions. Knowledge of these isolate-specific responses to the environment will lead to enhanced predictive capabilities for *Alexandrium* bloom dynamics and PSP events. The ultimate goal is to develop temporal and spatial maps of potential *Alexandrium* toxicity that will aid monitoring programs in targeting specific areas likely to be PSP hotspots.

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Relationship of *Pfiesteria* spp. and *Pfiesteria*-like Organisms to Environmental Factors in Tidal Creeks Draining Urbanized Watersheds

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Abstract

During a 1997 survey of a set of urbanizing tidal creeks, the abundance of *Pfiesteria*-like dinoflagellates (PLOs) was correlated with chlorophyll *a*, nitrate, and total phosphorus concentrations. Subsequent PCR surveys in 2000 and 2002 verified the presence of *Pfiesteria piscicida* and *P. shumwayae* in the two most eutrophic creeks, demonstrating that urban nutrient runoff can create conditions conducive to *Pfiesteria* and PLO propagation.

Introduction

The tidal creek complex in New Hanover County, North Carolina, drains a rapidly urbanizing area. Thus, some areas within the creeks receive anthropogenic nutrient loading that leads to dense phytoplankton blooms, sometimes exceeding 200 µg chlorophyll *a*/L (Mallin *et al.,* in press). The estuarine salinities, nutrient inputs, and abundant phytoplankton prey (especially cryptomonads) make these creeks potentially suitable habitat for *Pfiesteria* spp. (Burkholder *et al.,* 2001). During 1996 in Hewletts Creek, which drains a heavily developed watershed, we found a massive bloom of cryptomonads (13,000 cells/mL) being grazed by abundant *Pfiesteria*-like dinoflagellates (2,600 cells/mL; Mallin *et al.,* 1999).

In contrast, tidal creeks in relatively pristine areas in South Carolina have been shown to host low abundances of *Pfiesteria* spp. (Lewitus *et al.,* 2002). The low *Pfiesteria* spp. densities were believed to be a function of low phytoplankton prey abundance resulting from low inorganic nutrient inputs. The urbanized New Hanover County tidal creek complex provides a similar physical environment to the South Carolina creeks, but urban non-point source runoff provides direct nutrient loading. *Pfiesteria*-linked fish kills have not been reported in the New Hanover County tidal creeks. However, the designation of these tidal creeks by the State of North Carolina as primary nursery areas for finfish makes the potential presence of *Pfiesteria* and other harmful algae a special concern. Here we report the data from surveys of this tidal creek complex for *Pfiesteria* spp. and related organisms.

Materials and Methods

In summer 1997 we conducted a *Pfiesteria* survey by collecting phytoplankton samples at stations throughout Bradley, Hewletts, Howe, Pages and Futch creeks. The sampling locations encompassed a spectrum of nutrient concentrations, phytoplankton abundances, and salinities. Along with phytoplankton samples, we measured physical parameters, nutrients, and chlorophyll *a*. Samples were collected in May, June, July, August, and September, at or near high tide. Vertical profiles were taken for dissolved oxygen, turbidity, pH, temperature, and salinity using a YSI 6820 sonde linked to a YSI 610 display unit.

Presumptive counts (flagellated stages of *Pfiesteria* and *Pfiesteria*-like organisms (PLOs); Burkholder and Glasgow, 1997; Glasgow *et al.,* 2001) from the acidic Lugol's-preserved phytoplankton samples (as in Burkholder and Glasgow, 1997) were performed by light microscopy (600×, phase contrast). Duplicate samples were collected for nitrate + nitrite (hereafter referred to as nitrate), orthophosphate, total nitrogen, total phosphorus, and ammonium, and were analyzed using a Technicon AutoAnalyzer. Triplicate chlorophyll *a* samples were filtered through 1.0 µm pore-sized glass fiber filters. The filters were frozen for 12–24 hours, extracted in 10 mL of 90% basic acetone, and analyzed for chlorophyll *a* using a Turner AU-10 fluorometer.

We performed correlation analyses to test for relationships between PLO abundance and environmental factors. The parameters were first tested for normality using the Shapiro-Wilk test (Statistical Analysis System, SAS Institute, Inc.). The physical parameters proved to be normally distributed, whereas nutrients, chlorophyll *a*, and PLO counts required log transformation before correlation analyses were performed.

In October 2000 we collected samples at five of the previous locations to test for the presence of *Pfiesteria piscicida* Steidinger and Burkholder and *P. shumwayae* Glasgow and Burkholder using the polymerase chain reaction (PCR; Rublee *et al.,* 2001). These samples were later reanalyzed by a more sensitive real-time PCR protocol using Taqman probes (Bowers *et al.,* 2000). In July 2002 we collected samples at 11 stations throughout the creeks at both low tide and high tide, which were also analyzed by real-time PCR.

Results and Discussion

Results of the 1997 Survey A total of 65 samples were collected, and PLOs were found in four samples from upper Bradley Creek, two samples in upper Pages Creek, and one sample each in upper Hewletts and Howe Creeks, with abundances ranging up to 750 cells/mL in Bradley Creek. PLOs were found in June, July and August, but not in May or September. Sites where PLOs were found were more nutrient-enriched than non-PLO sites, and chlorophyll *a* was fourfold higher in PLO areas than non-PLO areas (Table

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Table 1 Comparison of environmental conditions in tidal creek sites, with PLOs versus without PLOs, in summer 1997. Data are given as means ± 1 standard deviation, and range.

Parameter	Sites without PLOs	Sites with PLOs
salinity (psu)	27.8 (8.7), 0–36.5	15.7 (13.8), 0.0–33.8
ammonium-N (mg/L)	36 (25), 5–145	31 (13), 13–53
nitrate-N (mg/L)	33 (87), 1–582	39 (26), 4–84
total nitrogen (mg/L)	384 (178), 153–1,406	457 (170), 212–814
orthophosphate-P (mg/L)	8 (5), 1–28	10 (9), 1–28
total phosphorus (mg/L)	51 (18), 11–105	72 (40), 33–165
chlorophyll *a* (mg/L)	9 (7), 2–31	33 (32), 8–109

1). Salinity was considerably lower in PLO areas, with average salinity at approximately 16 psu. PCR analyses conducted in 2002 on preserved samples from the 1997 survey verified the presence of *P. shumwayae* in anthropogenically impacted Bradley Creek.

There was a strong positive correlation between PLO abundance and chlorophyll *a* (Table 2). There were also significant positive correlations between PLO abundance and nitrate and total P (Table 2). In contrast, a strong negative correlation was found between PLO abundance and salinity.

Results of 2000 and 2002 PCR Surveys In 2000, the presence of *P. shumwayae* was detected by PCR analysis in two samples in Bradley and Hewletts creeks, two of the most eutrophic creeks (with the most highly urbanized watersheds) within the tidal creek complex. During the 2002 low tide sampling, *P. piscicida* was found by PCR in Bradley Creek (coinciding with chlorophyll *a* at 160 µg/L). Microscopic examination of the phytoplankton samples revealed very few *Pfiesteria*-like dinoflagellates, despite high chlorophyll *a* concentrations in several other locations. PCR analysis of high tide samples detected the presence of *P. piscicida* in upper Hewletts Creek only.

The correlation between the abundance of *Pfiesteria* and PLOs and chlorophyll *a* and nutrients suggests that *Pfiesteria* and related dinoflagellates prefer eutrophic environments. The PCR surveys showed *Pfiesteria* spp. only in Hewletts and Bradley Creeks, the most eutrophic tidal creeks in the region. This supports other work (Burkholder and Glasgow, 1997; Burkholder *et al.,* 2001; Glasgow *et al.,* 2001; Glibert *et al.,* 2002) describing strong associations between *Pfiesteria* and nutrient-enriched areas of larger estuaries in North Carolina and Chesapeake Bay.

Pfiesteria spp. have shown positive responses to nutrients in laboratory experiments (Burkholder and Glasgow, 1997; Lewitus *et al.,* 1999a). These organisms respond positively to enrichment in several ways. *Pfiesteria* exhibits kleptoplasticity, or the sequestering of chloroplasts from prey phytoplankton (Lewitus *et al.,* 1999b). When it has kleptochloroplasts, it can respond positively to inorganic nutrient enrichment as a mixotroph (Burkholder and Glasgow, 1997; Lewitus *et al.,* 1999a; Burkholder *et al.,* 2001). As a heterotroph, *Pfiesteria* responds positively to increases in algal prey that increase along with nutrients (Glasglow *et al.,* 2001), and it can take up organic nutrient forms directly (Burkholder and Glasgow, 1997, Lewitus *et al.,* 1999a).

Low *Pfiesteria* abundances reported in South Carolina tidal creeks have been attributed to low phytoplankton prey abundance, which is limited by the low nitrate levels (Lewitus *et al.,* 2002). Nitrate is the primary nutrient that limits phytoplankton productivity in the urbanized tidal creeks in southeastern North Carolina (Mallin *et al.,* in press). These creeks receive nitrate and other nutrients from golf courses, suburban lawns and gardens, landscaped areas, and pet waste deposited in suburban and urban areas (Mallin and Wheeler, 2000). In response to these nutrient pulses, phytoplankton blooms occur (Mallin *et al.,* in press), often consisting largely of cryptomonads (Mallin *et al.,* 1999), which are a known food source for *Pfiesteria* spp. (Burkholder *et al.,* 2001).

The two creeks where *Pfiesteria* spp. were detected by PCR in 2000 and 2002 have the two most developed watersheds in the tidal creeks system (Mallin *et al.,* 1999; in press). Abundance of *Pfiesteria* spp. has been linked to elevated nutrients delivered to estuaries through municipal and industrial wastewater outfalls (Burkholder and Glasgow, 1997; Burkholder *et al.,* 2001), and concentrated animal feeding operations and other agricultural areas (Burkholder and Glasgow, 1997; Glasgow *et al.,* 2001). This study indicates that urban runoff of nutrients into small estuaries can also provide appropriate conditions for the

Table 2 Results of correlation analyses between PLO abundance and environmental factors in 1997. Only statistically significant relationships ($a = 0.05$) are shown (Pearson correlation coefficient R / probability [*P*]).

Parameter	Salinity	Nitrate-N	Total *P*	Chlorophyll *a*
PLO abundance	–0.495	0.289	0.284	0.497
	0.001	0.020	0.022	0.001

propagation of *Pfiesteria* spp. and related organisms.

Based on these surveys, there may be lesser numbers of *Pfiesteria* and *Pfiesteria*-like species in this creek system in 2002 relative to observations in 1996 (Mallin *et al.,* 1999) and the survey in 1997, although nutrients and chlorophyll *a* concentrations remain high. Following the 1997 survey, this region experienced several hurricanes, including Bonnie in 1998, and Dennis, Floyd and Irene in 1999. Results from research in other local estuaries indicated that high river discharge displaced *Pfiesteria* spp. into the sounds and other coastal waters (Burkholder *et al.,* 2001). We hypothesize that *Pfiesteria* cysts in the tidal creek sediments were strongly impacted by flushing during these events, reducing the cyst reservoir of *Pfiesteria* spp. However, detection of *Pfiesteria* spp. 1–3 years after the hurricane events suggests that populations of these organisms are somewhat resilient to massive flooding impacts. Because of the continued inputs of nutrients into the tidal creeks, these populations will likely recover in time.

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Flow Cytometry Techniques to Quantify Prey Species of *Pfiesteria*-Like Dinoflagellates

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Abstract

An understanding of predator-prey relationships is crucial to assess the role of *Pfiesteria*-like dinoflagellates as grazers upon autotrophic microalgae. Experiments involving comparative grazing on several, simultaneously available prey species may best model natural systems. Enumeration of statistically sufficient numbers of predators and multiple prey species in such a setting renders manual counting methods impractical. Flow cytometry is ideally suited to experiments involving mixed phytoplankton populations because of 1) high sample throughput, 2) the capacity for near real-time analysis of live or fixed cells and 3) statistically robust data often based upon tens of thousands of individual measurements. Equivalent biovolumes of prey were introduced into cultures of a *Pfiesteria*-like dinoflagellate. Fixed samples collected at time points after prey introduction were then analyzed by flow cytometry. Fluorescent signatures were utilized to identify and quantify subpopulations of predators and prey. When compared to controls, a measurable decrease in prey density was attributed to grazing by dinoflagellates.

Introduction

Phagotrophic dinoflagellates represent an important component of marine, estuarine and freshwater food webs (Gaines and Elbrächter, 1987; Jeong, 1999). Among these phagotrophs, *Pfiesteria*-like species have become recognized as potentially important estuarine micrograzers with a global distribution (Jakobsen *et al.,* 2001; Rublee *et al.,* 2001). Like many systems, estuaries along the coast of eastern North America are often characterized by a wide variety of potential prey for micrograzers (Pinckney *et al.,* 1998; Mallin *et al.,* 1991). Therefore, experiments in which micrograzers are exposed to a single prey species do not adequately mimic the natural systems they seek to model. Although numerous studies have focused upon grazing by heterotrophic dinoflagellates, few attempts have been made to quantify grazing in mixed populations of prey.

Unfortunately, methods that can quantify grazers, together with multiple species of prey, are extremely limited. Manual counting is tedious, time-consuming, and often results in highly variable data. Most electronic particle counters do not provide adequate resolution when cells are of similar size or volume. In this study, we report development of a method to simultaneously quantify phagotrophic dinoflagellates and multiple prey species using flow cytometry. The results represent a preliminary component of ongoing research.

Materials and Methods

A *Pfiesteria*-like dinoflagellate, R3 (Fig. 1), was isolated in May 2001 from the Elizabeth River in Norfolk, Virginia, USA. The dinoflagellate was maintained in sterile Gulf Stream water diluted to a salinity of 15‰ and was incubated at 22 ± 0.5°C in 250 mL polystyrene tissue culture flasks. Moderate illumination (ca. 25 µmol photons · m^{-2} · s^{-1}) was provided by a bank of Sylvania Daylight™ 20-W fluorescent bulbs (Osram Sylvania, Danvers MA, USA) on a 14:10 light:dark cycle. The cryptophyte, *Rhodomonas* sp. (CCMP1319), was provided as a food source at 2–3 day intervals. *Rhodomonas* sp., the chrysophyte *Isochrysis galbana* and the chlorophyte *Dunaliella tertiolecta* (CCMP1320) were grown at 25 ± 0.5°C in F/2-Si (S = 30) under 80 ± 5 µmol photons · m^{-2} · s^{-1} of light with a 14:10 light:dark cycle. A small amount of *D. tertiolecta, I. galbana* and *Rhodomonas* sp. were added to a 100 mL culture of R3 and were incubated for two days at 23 ± 0.5°C under 25 µmol photons · m^{-2} · s^{-1} of light (12:12 light:dark cycle). A control culture containing only prey was also prepared. Two mL aliquots were removed from each culture at the beginning and end of incubation, were fixed with 1% glutaraldehyde and were stored in the dark at 4°C. Before

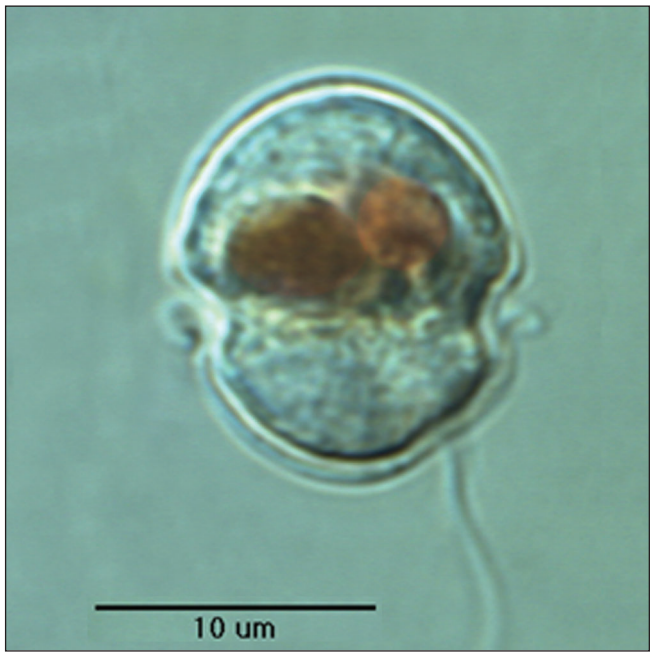

Figure 1 *Pfiesteria*-like dinoflagellate R3. The red-pigmented inclusions are food vacuoles containing the cryptophyte *Rhodomonas* sp.

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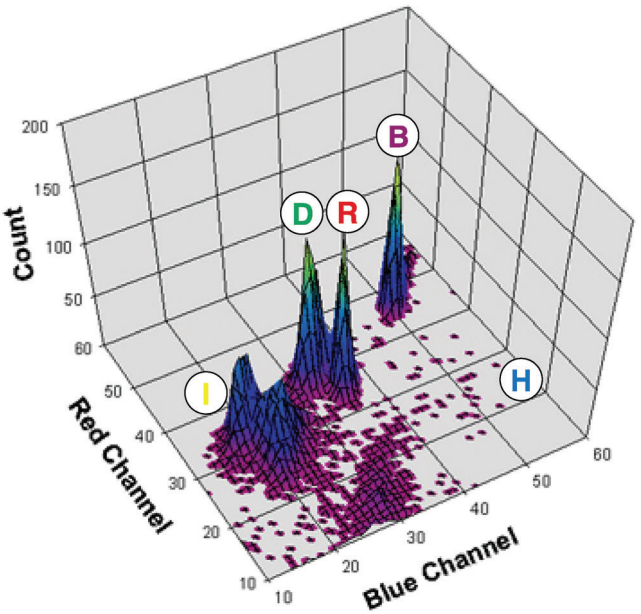

Figure 2 Flow cytometry data from grazing experiment with (H) the heterotrophic dinoflagellate R3, (I) *Isochrysis galbana,* (R) *Rhodomonas* sp., and (D) *Dunaliella tertiolecta,* as well as (B) fluorescent beads.

analysis, 1.0 µg mL^{-1} of DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate) (Sigma Chemical Co., St. Louis, MO, USA) and a known number of fluorescent beads (FlowCount™ beads, Beckman Coulter, Inc.) were added to each tube. A Coulter Epics Elite flow cytometer was utilized for sample analysis after the instrument was aligned with FlowChek™ calibration beads (Beckman Coulter, Inc.). The instrument was equipped with an Enterprise II™ laser, with peak emission bands centered at 488 nm and 358 nm. The standard optical filters supplied with the flow cytometer were replaced with a suite of filters designed to better resolve red, orange and blue emission wavelengths. Samples were quantified such that data acquisition stopped when either 1000 or 1500 beads were detected. Relative numbers of dinoflagellates and each prey species were determined using empirically determined analysis gates. The prey species were of very similar size and shape, and each contained chlorophyll *a*. Therefore, forward scatter, side scatter, or red fluorescence alone failed to provide adequate resolution to quantify populations of cells. A combination of red and blue fluorescence was found to allow the greatest degree of separation among all prey species. This combination also allowed dinoflagellates to be identified by their large DAPI-labeled nuclei and red fluorescent food vacuoles. Cell concentrations were calculated using the product of cells:beads and the number of beads mL^{-1}. Resulting data were analyzed using the software provided with the instrument.

Results and Discussion

The custom filter set combined with DAPI labeling allowed discrimination between heterotrophic dinoflagellates and each prey species (Fig. 2). Populations of *D. tertiolecta* and *Rhodomonas* sp. were identified as adjacent peaks near the center of the graph, while *I. galbana* exhibited a distinct bimodal distribution farther down the blue axis (see Fig. 2). The identity of each population in flow cytograms was confirmed using unialgal samples of each species. Dinoflagellate cells were loosely distributed over the upper end of the blue axis. Although microscopy confirmed ingestion of cryptophytes, dinoflagellate growth was fairly low (k = 0.277), suggesting that none of the prey were adequate to supply the requirements of the heterotrophs. Growth rates and grazing coefficients (Table 1) were calculated using the equations given by Frost (1972). The dinoflagellate exhibited the highest grazing rate relative to *Rhodomonas* (g = 0.251), while the lowest was calculated for *Isochrysis* (g = -0.0198). Even after two days, no dinospores were observed with green or yellow vacuoles indicative of ingestion of *D. tertiolecta* or *I. galbana,* respectively. This pattern suggests an apparent preference for *Rhodomonas* sp. by the dinoflagellate. However, the decline in *Rhodomonas* population cannot necessarily be attributed to a preference by R3. The strain of *Rhodomonas* provided was used to maintain R3 in culture for many months prior to the experiment. Therefore, R3 may have been "conditioned" to consume this particular cryptophyte. Secondly, the experiment was not designed to test for interactions between prey species. Without adequate controls, alleopathic interactions and competition between strains are likely to complicate or even mask grazing rates. Third, this study represents an unreplicated preliminary experiment addressing the feasibility of the counting methods employed. With no replication, simple variability between culture flasks and the effect upon apparent rates of grazing were not addressed. Nonetheless, the results do suggest that flow cytometry can be successfully applied to growth or grazing studies using multiple species of prey.

When combined with careful filter selection and the use of cellular markers such as DAPI, flow cytometry offers advantages over other methods of cell counting. Flow cytometric methods allow rapid processing and analysis of samples. Tens of thousands of cells from each sample can be easily counted, thereby increasing the accuracy and statistical strength of the dataset. More than 50 samples can

Table 1 Results of grazing experiment showing (k), the growth rate of each species in the control flask; (k*), the growth rate of each species in the flask with dinoflagellates added; and (g), the Grazing Coefficient defined as k–k*. Abbreviations follow Frost (1972).

Species	k (d^{-1})	k* (d^{-1})	g (d^{-1})
Rhodomonas sp.	0.0647	–0.186	0.251
Dunaliella tertiolecta	0.0505	0.0126	0.0379
Isochrysis galbana	–0.261	–0.241	–0.0198
Combined prey	–0.0976	–0.149	0.0509
R3	0.277		

readily be processed in a single day, yielding very high sample throughput. Disadvantages include high instrument cost and difficulty with data interpretation. Furthermore, periodic confirmation of flow cytometric data with manual counts is an important quality control step that cannot be overlooked. Further research with this method is needed to better characterize prey preference in phagotrophic dinoflagellates in the presence of a suite of potential prey.

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Chronic Urea Loading: A Correlate of *Pfiesteria* spp. in the Chesapeake and Coastal Bays of Maryland, USA

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Abstract

Since the first outbreaks of *Pfiesteria* spp. in the tributaries of the Chesapeake and Coastal Bays of Maryland, USA, a relationship with nutrients from agriculture and intensive animal (poultry) operations has been hypothesized. As an index of these inputs, concentrations of the nutrient urea were examined at >18 stations for several years. Urea is both a commonly used nitrogen fertilizer and is a decomposition product of poultry manure. Distribution of *Pfiesteria* spp. in the sediment and in the water column was also surveyed. The average urea concentration was found to be an excellent predictor of the percent positive detection for *Pfiesteria* spp. in the sediment ($R^2 \geq 0.94$). The correlation with water column *Pfiesteria* spp. presence was also positive but was weaker due to lower overall abundance and the transient nature of the zoospores. The enriched sites had mean concentrations of urea >1.0 µg at N L^{-1}, and seasonally pulsed concentrations >5 µg at N L^{-1}. These findings underscore the association of *Pfiesteria* spp. with organic-rich, nutrient- polluted waters. The role of nutrients in *Pfiesteria* growth may either be via direct uptake, or via indirect stimulation of other phytoplankton or bacteria on which *Pfiesteria* may graze.

Introduction

The presence of *Pfiesteria* was first documented in Chesapeake Bay in 1992 (Lewitus *et al.,* 1995), but the first major outbreaks were documented in 1997 (Burkholder and Glasgow, 1997; Magnien, 2001a). Since that time, an intensive monitoring program has been undertaken to record the environmental conditions of the bay and the presence of *Pfiesteria,* using molecular probe techniques. *Pfiesteria* spp. has been found to occur in several tributaries annually, but fish health events have been few in number (Magnien, 2001b).

Since the first documented outbreaks of *Pfiesteria* in both the Chesapeake Bay and the Neuse Estuary, North Carolina, the correlation with high levels of nutrients has been suggested (Burkholder and Glasgow, 1997), and the relationship between *Pfiesteria* and nutrients derived from agricultural and animal operations has been the subject of considerable debate in environmental management. The Chesapeake and Coastal bays of Maryland receive nutrients from agricultural runoff and from point-source discharges. Agricultural inputs of nutrients on Maryland's eastern shore are rich in both phosphorus and nitrogen in inorganic and organic forms (Boynton *et al.,* 1982; Glibert *et al.,* 2001). In contrast, on the western shore of the Chesapeake Bay, the majority of nutrients are delivered from point source discharges, primarily from the metropolitan regions of Baltimore and Washington, D.C. (Boesch *et al.,* 2001). Many organic nutrients are not typically reported in nutrient monitoring programs. As part of a larger study of the relationship between nutrients and *Pfiesteria* spp., in this report we explore the relationship with one agricultural nutrient, urea. Urea is used as an agricultural nitrogen fertilizer, and it is also a decomposition product of poultry manure, another commonly applied fertilizer. Urea has also been shown to be one of several nitrogen forms taken

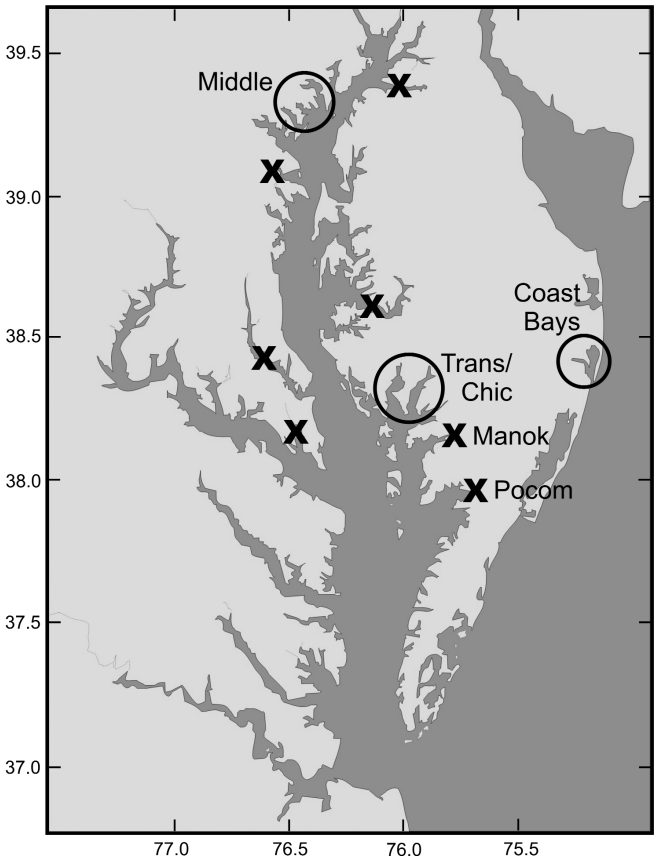

Figure 1 Map of Chesapeake Bay and Coastal Bays of Maryland indicating general regions of sampling. Regions that are circled had more frequent positive identifications of *Pfiesteria* spp. Regions indicated by an **X** had significantly fewer observations of *Pfiesteria*. Middle River, Transquaking (Trans), Chicamacomico (Chic), Manokin (Manok) and Pocomoke (Poco) rivers, as well as the Coastal Bays, are indicated on the map.

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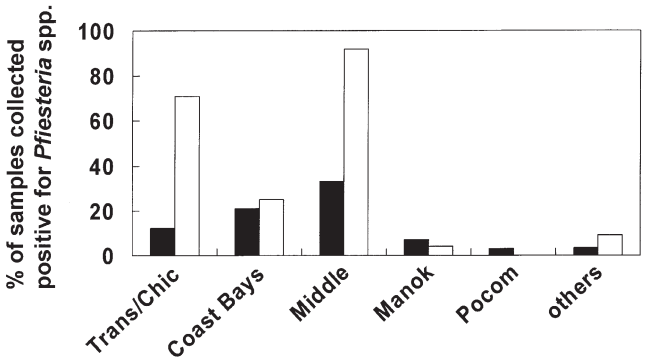

Figure 2 Percent of samples collected in water (solid bars) and in sediment (open bars) that were positive for *Pfiesteria* spp. in the Transquaking/Chicamacomico rivers (Trans/Chic), Coastal Bays, and Middle, Manokin (Manok) and Pocomoke (Pocom) rivers.

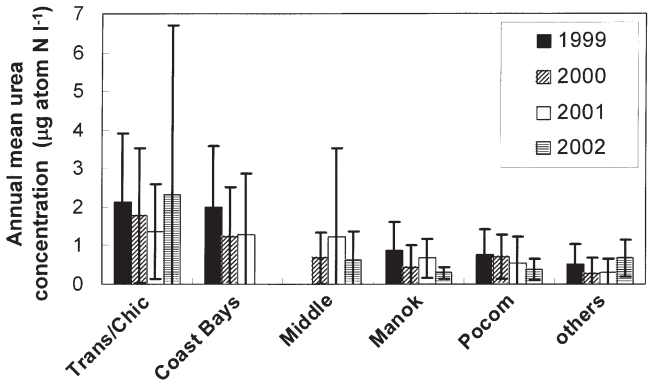

Figure 3 Annual mean concentration of urea for each of the regions and years shown. Error bars are ±1 SD.

up directly by *Pfiesteria* (Lewitus *et al.,* 1999b).

Pfiesteria spp. are not algae, but rather "heterotrophic" dinoflagellates that primarily depend on grazing to meet their nutritional requirements. It has previously been documented that *Pfiesteria* can graze on bacteria, algae, and bits of fish tissue (Burkholder and Glasgow, 1997; Burkholder *et al.,* 2001). *Pfiesteria* also has the ability to retain chloroplasts from algae that it grazes and can photosynthesize for short periods of time with these intact organelles (Lewitus *et al.,* 1999a,b). *Pfiesteria* spp. have the ability to take up dissolved nutrients directly, albeit at rates significantly slower than would be required to meet the entire growth demand of the cells (Lewitus *et al.,* 1999b). Outbreaks of *Pfiesteria* spp. in Chesapeake Bay tributaries have also been shown to be associated with high organic nitrogen and carbon (Glibert *et al.,* 2001). Here we document exceptionally high concentrations of urea in eastern shore tributaries, and a correlation between high urea loading and the presence of *Pfiesteria* spp. This relationship underscores the notion that *Pfiesteria* spp. is most common in organic-rich waters.

Materials and Methods

Two types of sampling were undertaken. As part of the established monitoring program for environmental variables in the Chesapeake and Coastal bays and their tributaries, urea concentrations were measured at 18–27 stations on a biweekly or monthly basis from April through October during the years 1999–2002 (n = 243 for 1999; 257 for 2000; 272 for 2001; 122 for 2002; Fig. 1) concomitant with other nutrients and water quality parameters (not reported here). Water samples for urea analysis were collected, filtered, then frozen for later analysis (within about 3 weeks; Parsons *et al.,* 1984). Sediment and water samples for spp. identification (n = 1,614 for water and 156 for sediment; Magnien *et al.,* 2002) were collected in 2000 and 2001, extracted for DNA, then analyzed by Taqman-based PCR assays specific for *P. piscicida* and *P. shumwayae* (Bowers *et al.,* 2000).

Results and Discussion

Of the sites regularly monitored in Maryland, *P. piscicida* and *P. shumwayae* are most frequently observed in the Transquaking, Chicamacomico, and Middle Rivers, and in the Coastal Bays (Fig. 2). *Pfiesteria* spp. were more frequent in sediment samples than in water samples, reflecting the benthic life stages of this organism as well as the transient nature of zoospore stages.

Annual mean concentrations of urea were significantly higher (>1 µg at N L^{-1}) in the same tributaries where *Pfiesteria* sp. were regularly observed: the Transquaking, Chicamacomico and Middle rivers, and in the Coastal Bays (Fig. 3). Maximum observed concentrations of urea for these sites were up to tenfold higher than annual means (not shown). For comparison, concentrations of urea along the main axis of Chesapeake Bay typically do not exceed 1 µg at N L^{-1} in surface waters (Lomas *et al.,* 2002). Focusing on the years 2000 and 2001, the years for which these reported *Pfiesteria* surveys were conducted, both average urea concentration and the average number of days for each tributary during which urea concentration exceeded 1.5 µg at N L^{-1} were found to be excellent predictors of the percent of sediment samples found to be positive for *Pfiesteria* spp. (Fig. 4A,B). For each relationship, the R^2 was ≥0.94 and was significant at $P < 0.01$. The relationships between urea concentration or number of days with elevated concentration and the percent of samples positive for *Pfiesteria* spp. in the water column were also positive (R^2 = 0.32–0.36) but were not significant. As *Pfiesteria* spp. is primarily a benthic organism, a weaker relationship with water column presence is not surprising. Presence in the sediment indicates the potential for an outbreak should other conditions be suitable for its proliferation.

This report builds on previous evidence that organic nutrients may be good predictors of *Pfiesteria* spp. Glibert and Terlizzi (1999) observed that dinoflagellate blooms, including *Pfiesteria* spp., in fish aquaculture ponds co-occurred with elevated concentrations of urea. In the Chesapeake Bay, *P. piscicida* was found to occur under conditions of elevated organic carbon, and the ratio of

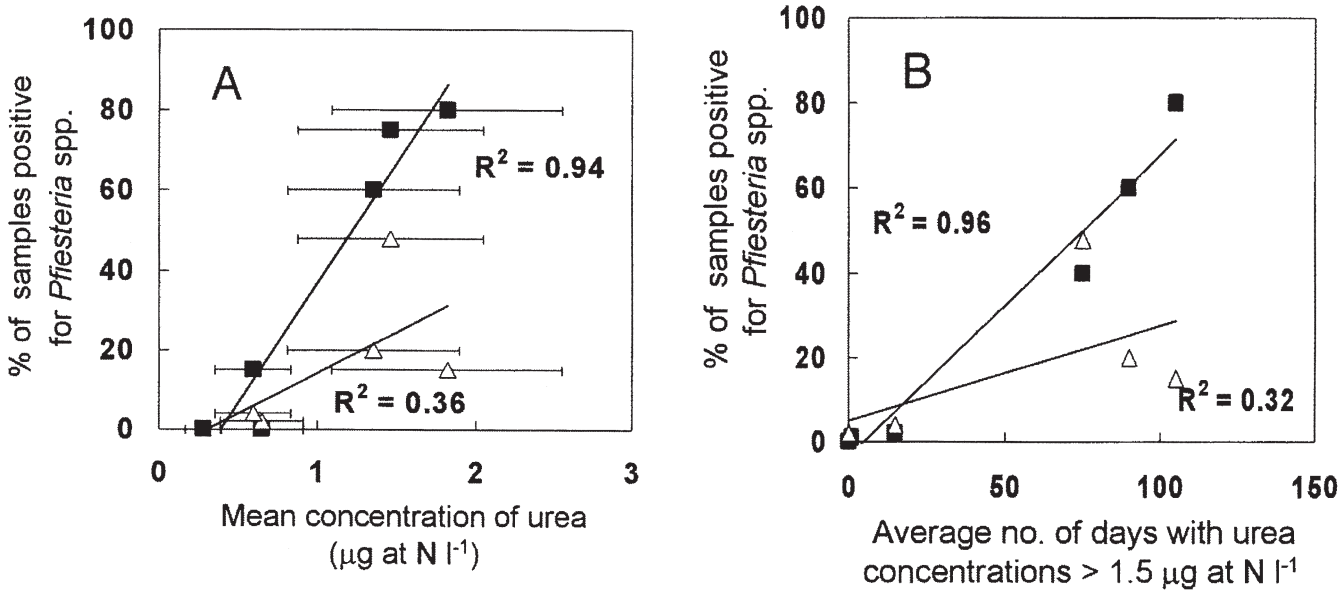

Figure 4 **A** Correlations between mean concentration of urea in Maryland eastern shore tributaries during 2000–2001 and the percent of samples positive for *Pfiesteria* spp. in the sediment (squares) and the water column (triangles). Error bars are ±1 SD. **B** Correlations between the average number of days (Apr–Oct) with urea concentrations in excess of 1.5 µg at N L^{-1} and the percent of samples positive for *Pfiesteria* spp. in the sediment (squares) and the water column (triangles).

dissolved organic carbon to dissolved organic nitrogen was a better predictor of *P. piscicida* than any inorganic nutrient (Glibert *et al.,* 2001). Locations with higher percentages of samples positive for *Pfiesteria* spp. in the sediment samples reported here were also found to have higher sediment nitrogen, phosphorus and carbon than locations that tested negative for *Pfiesteria* presence (Magnien *et al.,* 2002). Species with a complex nutrition such as *Pfiesteria* spp. may be stimulated by the direct assimilation of dissolved nutrients but more likely by the development of the algal food on which they preferentially graze (Burkholder and Glasgow, 1997; Burkholder and Glasgow, 2001; Parrow *et al.,* 2001). Urea is not the only nutrient which may directly or indirectly stimulate *Pfiesteria,* but elevated concentrations appear to serve as a useful indicator of the presence of *Pfiesteria* spp. in Maryland's agriculturally-influenced tributaries.

Acknowledgements

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The Production of Hydrogen Peroxide by *Heterosigma akashiwo* Under Varying N:P Ratios

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Abstract

Heterosigma akashiwo is a raphidophyte that causes massive fish kills in many coastal waters. One of the proposed mechanisms of ichthyotoxicity for raphidophytes is the production of reactive oxygen species (ROS). This project involved studying *H. akashiwo* under different N:P ratios in order to determine if nutrient conditions influence the amount of H_2O_2 that is released. *Heterosigma akashiwo* was cultured in N:P ratios of 9:1, 18:1, and 36:1, and H_2O_2 was measured throughout the growth curve via a scopoletin-based fluorometric assay. The maximum amount of H_2O_2 produced occurred during the mid-log phase of the growth curve. The N:P ratio of 36:1 was optimal for growth but the nitrogen-limited (N:P = 9:1) cultures produced significantly more H_2O_2.

Introduction

The flagellate *Heterosigma akashiwo* is a raphidophycean HAB phytoplankton species. It is ubiquitous in coastal waters and was involved in massive fish kills (Yang *et al.,* 1995; Twiner and Trick, 2000). The mechanism for ichthyotoxicity is still under study, but evidence indicates that *H. akashiwo* releases reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydrogen peroxide H_2O_2 (Yang *et al.,* 1995; Kim *et al.,* 1999), and in the presence of reduced metals, the hydroxyl radical (^-OH) may be formed. These radicals can elicit an overactive response in the fish, leading to excess mucus secretion resulting in asphyxiation (Oda *et al.,* 1992). The ROS focus of this project was hydrogen peroxide because it is more stable than the other two species (Asai *et al.,* 1999) and is likely to be produced on a sustained basis over prolonged periods of time (Twiner *et al.,* 2001).

In addition to absolute concentrations, nitrogen and phosphorus in aquatic environments are often expressed as N:P ratios. Using environmentally realistic concentrations, hydrogen peroxide production at different ratios was studied for two reasons: 1) N:P ratios are very important regulators of phytoplankton abundance and community structure because each species of phytoplankton has an optimal nutrient ratio (Bulgakov and Levich, 1999), and 2) because novel phytoplankton blooms have been increasing over the past two decides and the shift in N:P ratios may play a major role in this increase (Smayda, 1990).

This project determined hydrogen peroxide production from the raphidophyte *H. akashiwo,* under different N:P ratios. The objective of this study was to determine if nutrient conditions had any effect on reactive oxygen species generated by *H. akashiwo.*

Materials and Methods

A clone of *H. akashiwo* isolated from a bloom in Texas was continuously maintained in 3-L Fernbach flasks containing 1.5L of 36‰ Erdshreiber media. The experimental cultures were pre-conditioned over a three-month period to the different N:P ratios: 9:1, 18:1, and 36:1 (atoms) prior to each experiment. All cultures had an initial phosphate concentration of 5 g/L while each separate culture had 37.5, 75 and 150 mg/L $NaNO_3$, yielding ratios of 9:1, 18:1 and 36:1, respectively. All experimental cultures were counted daily in replicates of 4 with a Beckman Coulter Multisizer IIe to obtain growth curves and subsamples were extracted every 3–4 days throughout the growth curve for hydrogen peroxide analysis. Cells were resuspended in

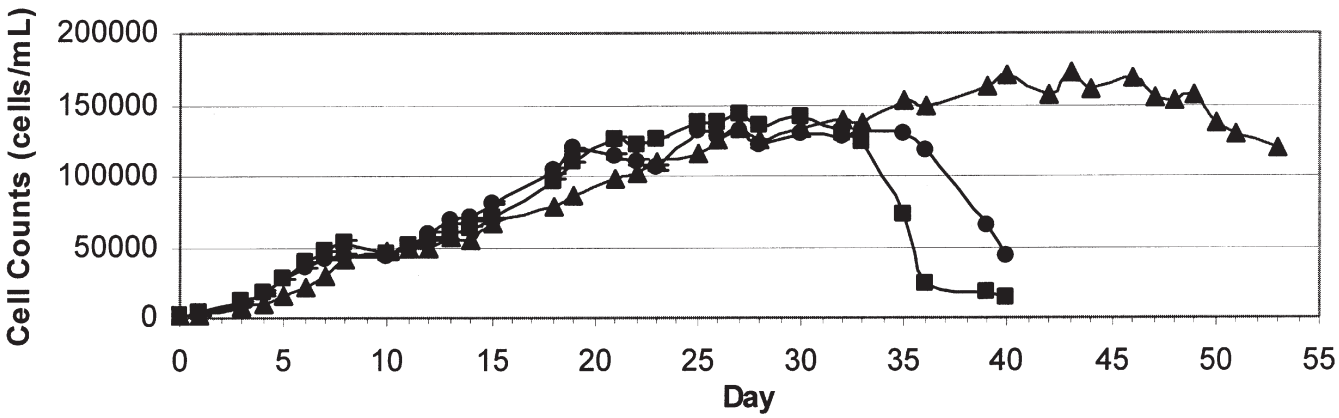

Figure 1 Growth curves for *Heterosigma akashiwo.* ● N:P = 9:1, ■ N:P = 18:1, ▲ N:P = 36:1.

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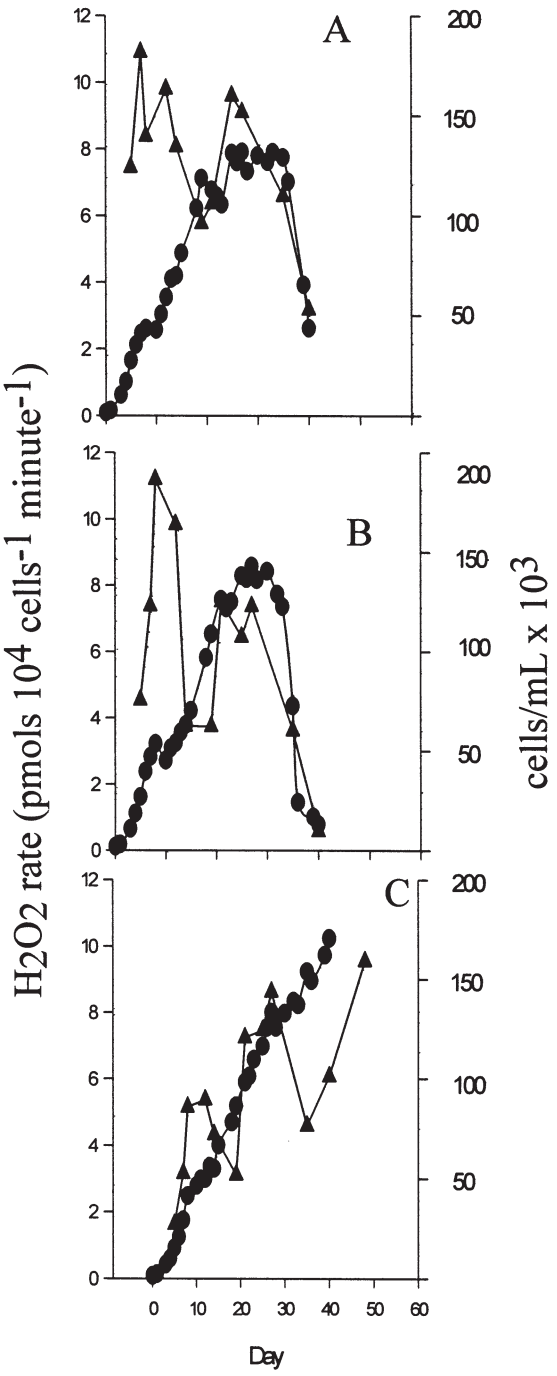

Figure 2 Growth curves and rate of H_2O_2 production for cultures grown in media with N:P ratios of **A** 9:1, **B** 18:1, and **C** 36:1. ● = cell counts, ▲ = H_2O_2 rate.

a peroxide-free seawater media, and hydrogen peroxide was analyzed using the horseradish peroxidase-mediated scopoletin fluorescence decay method (Cooper *et al.,* 1994, Kieber and Helz 1986). Standard curves for H_2O_2 in solution were performed prior to each series of analyses. The rate of H_2O_2, determined by measuring peroxide at t = 0 and again at t = 15 minutes and normalized on a per-cell basis from knowing the cell density of the resuspension, was calculated for each measurement. To determine whether the H_2O_2 production rate of one culture was significantly different from that of another, a one-way analysis of variance was performed, followed by a Tukey-Kramer comparison of all pairs. This analysis grouped together all of the replicates for each culture and compared them to all of the replicates of the other cultures. A positive number in the analysis indicated that there was a statistically significant difference between sets of cultures. In the Tukey-Kramer comparison, non-overlapping circles indicated a strong significant difference between sets of cultures.

Results and Discussion

In all three nutrient conditions, the cells obtained a maximum density of approximately 150,000 cells per mL. The cultures with N:P ratios of 9:1 and 18:1 declined at about day 40, while the culture with N:P = 36:1 did not decline until approximately Day 48 (Fig. 1). Based on these observations, it was concluded that of all nutrient conditions in this experiment, the N:P = 36:1 was capable of sustaining continued growth of *H. akashiwo.* The cells survived and perhaps even thrived under conditions that were presumably phosphorus-limiting. However, *H. akashiwo* is capable of storing phosphorus in the form of intracellular polyphosphate pools (Kimura *et al.,* 1999). The 36:1 culture appeared not to be limited by phosphate except at the very end of the experimental period. Phosphorus was still measurable in the media at that time.

A bimodal pattern of hydrogen peroxide production was observed in all three cultures (Fig. 2). One peak occurred in early log phase and the other occurred as the cells were entering the stationary phase of the growth curve. The amount of H_2O_2 produced by the *H. akashiwo* cells was substantial, up to 11.2 pmols per 10^4 cells per minute. Previous research proposed that H_2O_2 is produced photochemically and that biological sources are negligible (Cooper *et al.,* 1994, Palenik *et al.,* 1987;1989). However, the current study shows that in areas where raphidophyte blooms occurred, biological production of hydrogen peroxide could be a very significant source.

In two of three nutrient conditions, the maximum H_2O_2 production occurred in early log phase (Fig. 2). *H. akashiwo* releases reactive oxygen species within the first week of the bloom. Often, a bloom is not recognized and monitored until it is fully developed, implying that the damage done by these oxygen radicals could occur before samples are ever collected or bloom is noted.

The N:P ratio could affect the production of H_2O_2 from *H. akashiwo* either directly or indirectly. A direct effect would suggest a link to either the nitrogen or phosphorus as a precursor or stimulant to the production of H_2O_2 to occur. However, an indirect effect would suggest that the H_2O_2 production is caused by stress, which in turn is caused by the N:P ratios. A substance that is produced as a result of stress is a secondary metabolite. Another possibility is that the nutrients indirectly affect the H_2O_2 production by causing metabolism to function in an altered way, concluding that H_2O_2 is a metabolic byproduct rather than a secondary metabolite. For example, a culture that is limited

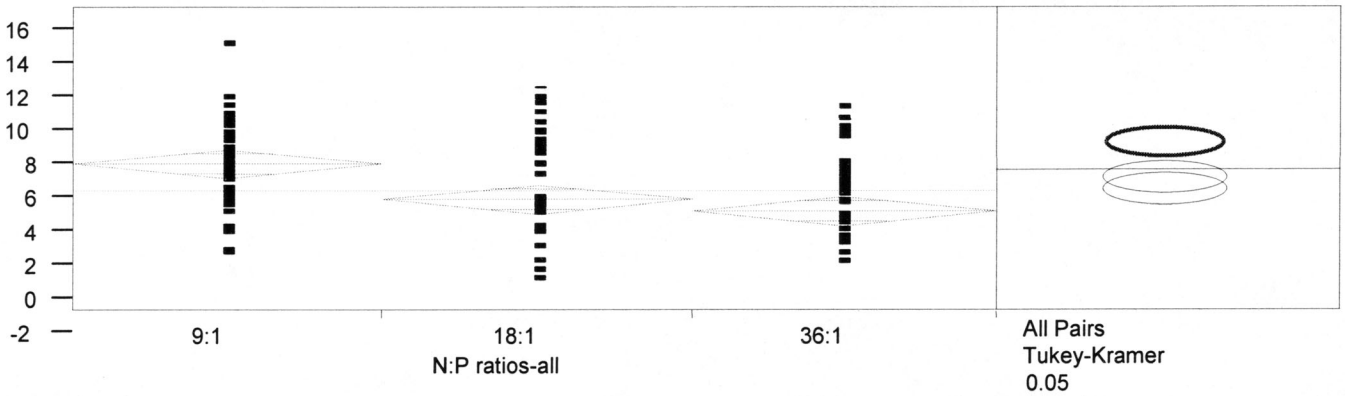

Figure 3 One-way ANOVA and Tukey-Kramer comparison analysis of all pairs for all days of H_2O_2 analysis.

by an essential element could cause H_2O_2 to be defectively released, whereas in healthy N:P ratios, the H_2O_2 is assimilated more efficiently.

The culture of *H. akashiwo* grown at an N:P ratio of 9:1 was the one that produced a significantly higher rate of peroxide than the other two cultures (Fig. 3). An N:P ratio of 9:1 created a situation of nitrogen limitation to the *H. akashiwo* cells. Nitrogen starvation leads to the activation of catabolic metabolism and the depression of carbon fixation during photosynthesis (Takahashi and Ikawa, 1988). Without sufficient nitrogen available, the *H. akashiwo* cells entered a state of stress and caused the cells to produce enhanced amounts of H_2O_2, either as a secondary metabolite or as a byproduct of faulty metabolism.

Acknowledgements

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Organic and Inorganic Nitrogen Uptake Kinetics in Field Populations Dominated by *Karenia brevis*

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Abstract

Blooms of the toxic dinoflagellate, *Karenia brevis,* are a persistent feature of the Florida Shelf, and are hypothesized to initiate in offshore oligotrophic waters. The development of blooms in this region, however, seems unlikely given the persistent low nitrogen (N) conditions. The overall goal of our project is to determine the source of N that fuels these high biomass accumulations of *K. brevis* on the Florida Shelf. One potentially important but unquantified source of new N to the area is inorganic and organic N regenerated by the N_2-fixing cyanobacterium, *Trichodesmium.* To quantify the ability of *K. brevis* to utilize a suite of N substrates, we performed two sets of kinetic experiments in the field at the height of a *K. brevis* bloom on the Florida Shelf in October 2001. At the time of the experiments, *K. brevis* dominated (>90%) the water column biomass with a maximum concentration of 9.5×10^6 cells L^{-1}. Short-term (1 hr) incubations were performed with ^{15}N-labeled substrates including NH_4^+, NO_3^-, and dissolved organic N (DON; urea, glutamate, and DON produced *in situ* during an earlier *Trichodesmium* bloom). Substrates were added at concentrations ranging from 0.055 to 200 µmol N L^{-1}. *K. brevis* was found to utilize all inorganic and organic N substrates offered. These data suggest that when high rates of N regeneration by *Trichodesmium* occur on the shelf, *K. brevis* has the physiological capabilities to efficiently exploit this source of N.

Introduction

Unpublished research, anecdotal information and historical red tide monitoring data suggest a correlation between the timing and magnitude of blooms of the toxic dinoflagellate, *Karenia brevis* (formerly *Gymnodinium breve*), and the occurrence of the filamentous, N_2-fixing cyanobacteria *Trichodesmium* spp. in the Gulf of Mexico and Atlantic coastal waters (Lenes *et al.,* 2001; Mulholland *et al.,* this Proceedings). We hypothesize that this correlation is due to a dependence of *K. brevis* on nitrogen (N) recently fixed and then released by *Trichodesmium.* Though there is a wealth of information on phosphorus utilization by *K. brevis,* relatively little is known about its N nutrition. Earlier work has shown that *K. brevis* is capable of utilizing NH_4^+ (Doig 1973) as well as a variety of amino acids and other organic N compounds as N sources (Wilson 1966; Baden and Mende 1979; Shimizu and Wrensford 1993). The objective of our study was to expand on this earlier work in order to quantify the affinity of *K. brevis* for different N substrates offered at a range of concentrations, including NH_4^+ and glutamate, both of which are known to be important release products of *Trichodesmium* (Capone *et al.,* 1994; O'Neil *et al.,* 1996).

Materials and Methods

Two sets of kinetic experiments were conducted with surface water (~1 m) in October in the Gulf of Mexico with water collected from a large bloom of *K. brevis* (see Heil *et al.,* this Proceedings). On October 2, 2001, kinetic parameters were measured for NH_4^+, NO_3^-, urea, and glutamate (glu). On October 4, 2001, kinetic parameters were measured for NH_4^+, NO_3^-, urea, and dissolved organic N (DON) that was produced *in situ,* during a cruise to the same area in July 2001, using concentrated *Trichodesmium* and ^{15}N-labeled N_2 gas (Glibert and Bronk 1994). Experiments were performed in 60-mL PETG bottles and incubated for ~1 hr in on-deck incubators. Each ^{15}N-labeled substrate was added at nine concentrations: 0.055, 0.1, 0.25 (replicated to provide an estimate of between bottle variability), 0.5, 1.0, 10, 50, 100, and 200 µmol N L^{-1}. Concentrations of ambient NH_4^+ (manual phenol-hypochlorate, Cochlan and Bronk 2001), NO_3^- (autoanalyzer), urea (manual monoxime, Cochlan and Bronk 2001), and dissolved free amino acids (DFAA), including glutamate (HPLC, Cowie and Hedges 1992), were determined at the start of incubations. Uptake rates were measured with ^{15}N tracer techniques, and ki-

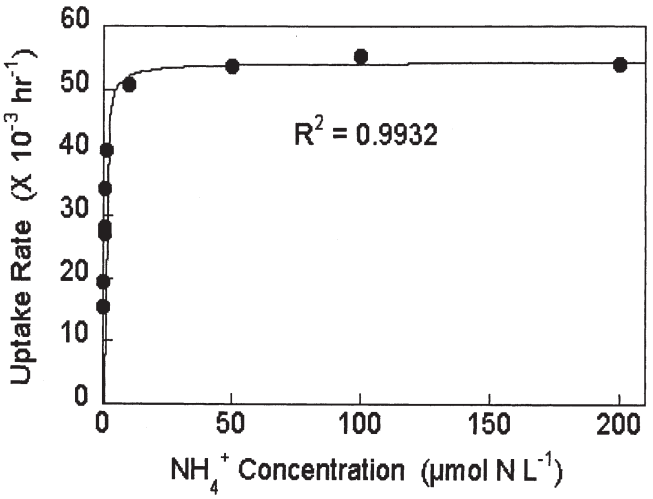

Figure 1 Rates of specific uptake of NH_4^+ as a function of NH_4^+ concentration.

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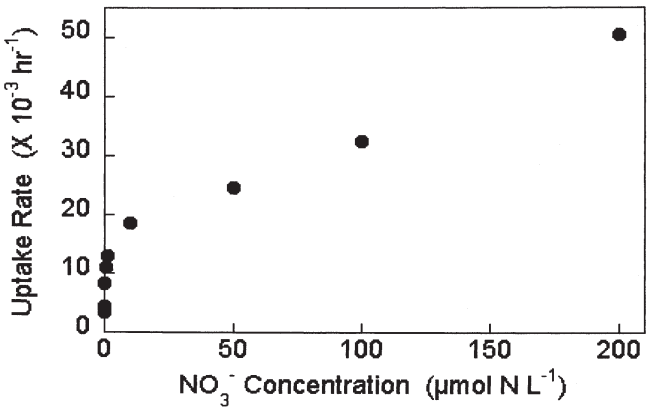

Figure 2 Rates of specific uptake of NO_3^- as a function of NO_3^- concentration.

netic parameters were estimated as in Cochlan and Bronk (2001).

Results and Discussion

During the first and second set of kinetic experiments, *Karenia* spp. accounted for 99.7% and 95.0% of phytoplankton cells, respectively; 99% of the *Karenia* were the species *K. brevis* on both dates (Heil *et al.,* unpub. data). Concentrations of NH_4^+ were low (0.04 to 0.15 µmol N L^{-1}) and NO_3^- concentrations were <0.05 µmol N L^{-1}. In contrast, organic N concentrations were relatively high; DON, urea, and DFAA concentrations were 13.0, 1.18–1.28, and 1.04 µmol N L^{-1}, respectively.

Uptake rates of NH_4^+ were related to substrate concentrations following Michaelis-Menten kinetics (Fig. 1). The K_s values for NH_4^+ were 0.41 and 0.29 for the two experiments performed. These K_s values are similar to the estimates made by Vargo (0.47, cited in Steindinger *et al.,* 1998). In the case of NO_3^-, however, uptake did not saturate even at 200 µmol N L^{-1} (not shown). In the lower portion of the curve (<10 µmol N L^{-1}), however, uptake was a hyperbolic function of substrate concentrations (Fig. 2). K_s values estimated only from this region of the curve were 0.06 and 0.33 µmol N L^{-1}1; these values are lower than the estimate of Vargo (0.42, cited in Steindinger *et al.,* 1998).

With respect to the organic N substrates, *K. brevis* was capable of utilizing all of the forms offered; three results are highlighted here. First, uptake of glutamate and urea did not exhibit Michaelis-Menten kinetics (Figs. 3, 4). At low substrate concentrations, there was an apparent increase in the specific uptake rates as substrate concentrations decreased. Eppley *et al.* (1977) also observed an apparent increase in urea uptake rates at the lowest substrate concentrations that were attributed to erroneously high determinations of ambient concentrations; in their study concentrations were near the limit of detection. In the present study, ambient concentrations of DFAA and urea were ~1 µmol N L^{-1}, which is high relative to the limit of detection and consistent with concentrations observed under these field conditions in the past. Ambient concentrations would have to have been near 0.05 µmol N L^{-1} for the curves to have been hyperbolic in shape, which is highly unlikely considering there was no evidence that anything was amiss analytically. It was also suggested that the lower atom % enrichments of the organic substrates, relative to the inorganic substrates, could have produced erroneously high uptake rates. The range of atom % enrichments that produced elevated glutamate and urea uptake rates were indeed lower than the atom % enrichments used in the NH_4^+ kinetics curve where no elevated uptake rates were observed (Table 1) so, as noted above, enrichment could have contributed to the elevated uptake rates at low concentrations. However, the range of atom % enrichments for the organics in this study is also within the range of substrate enrichments used in many other studies where no increase in uptake rates at low substrate concentrations was seen (*e.g.,* Cochlan and Bronk 2001). In addition, experiments have been done in the past where the concentration of substrate was held constant but the atom % enrichment was varied down to 0.01 atom % (Mulholland and Capone, unpub. data). No elevation in uptake rates was observed, even at the lowest enrichments tested. Additional work is clearly

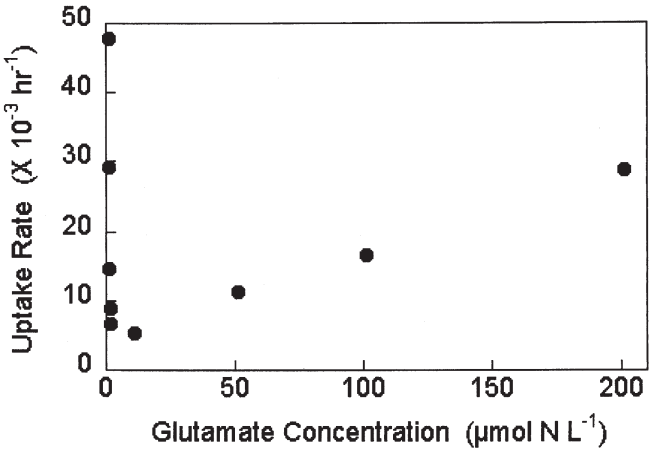

Figure 3 Rates of specific uptake of glutamate as a function of glutamate concentration.

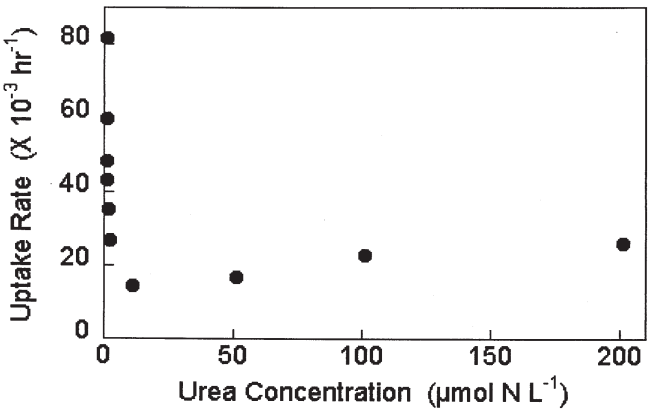

Figure 4 Rates of specific uptake of urea as a function of urea concentration.

Table 1 Size of ^{15}N additions (μmol N L^{-1}), atom % enrichments of the substrate pool (atom %), and uptake rates (uptake, μmol N L^{-1} h^{-1}) for urea, glutamate (glu) and ammonium (NH_4^+).

^{15}N Added	Urea Atom %	Urea Uptake	Glu Atom%	Glu Uptake	NH_4^+ Atom %	NH_4^+ Uptake
0.05	4.4	81.6	5.0	47.7	26.9	15.5
0.1	7.8	59.8	8.8	29.1	40.2	19.5
0.25	17.5	45.7	19.4	14.6	62.7	27.5
0.5	29.7	35.2	32.5	8.9	77.0	34.2
1.0	45.8	26.8	49.0	6.7	87.0	40.3
10	89.4	14.3	90.6	5.3	98.5	50.6

needed to determine the cause of the apparent elevated uptake rates at low substrate concentrations. Are these data an indicator of some unique physiology or simply an experimental artifact?

Second, glutamate uptake was not saturated at concentrations of 200 μmol N L^{-1} (Fig. 3), similar to the situation observed with NO_3^- (Fig. 2). Glutamate is an important release product of *Trichodesmium* (Capone *et al.,* 1994), such that concentrations of glutamate in the immediate vicinity of a *Trichodesmium* colony would likely be quite high. The ability to rapidly incorporate glutamate from such a highly enriched patch would be of considerable advantage to *K. brevis* cells in the Gulf of Mexico.

Third, and perhaps most importantly, *K. brevis* was found to be capable of utilizing ^{15}N-labeled DON produced as a result of the fixation of ^{15}N-labeled N_2 gas (Fig. 5). The DON present in the tracer was likely a combination of DFAA and possibly small proteins (Capone *et al.,* 1994, Bronk 2002). These data demonstrate *Trichodesmium*'s ability to produce organic matter that *K. brevis* can utilize at relatively low concentrations.

In conclusion, *K. brevis* was able to take up all inorganic and organic N substrates tested. The results of these kinetic experiments indicate that *K. brevis* is well adapted to meet its nutritional needs with a wide array of N substrates as they become available as well as under a wide range of substrate concentrations.

Acknowledgements

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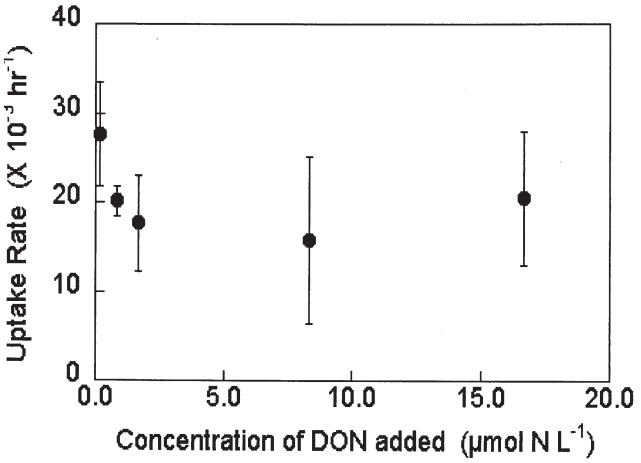

Figure 5 Specific uptake rates of DON produced *in situ* by *Trichodesmium* as a function of the concentration of DON tracer.

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A Massive Bloom of *Cochlodinium polykrikoides* in the Yatsushiro Sea, Japan in 2000

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Abstract

In summer of 2000, a massive bloom of a harmful dinoflagellate *Cochlodinium polykrikoides* (Dinophyceae) occurred in the Yatsushiro Sea, western Japan. This bloom caused mass mortalities of cultured fish valued at about 40 billion yen. In order to evaluate the mechanism for the bloom outbreak, the developmental process and the environmental characteristics were investigated. From 10 to 24 July, a bloom of *C. polykrikoides* occurred when water temperature and salinity ranged 24.5°–26.6°C and 32–33 psu, respectively. Dissolved inorganic nitrogen and phosphorus concentrations were high through the middle and bottom layers during the development period of the bloom. Low precipitation and high salinity were also closely related to bloom development. Diatoms and other flagellates were not observed throughout the bloom.

Introduction

The Yatsushiro Sea is located off the southwestern coast of Kyushu Island, Japan (Fig. 1). The southern area of the sea exhibits physical properties of offshore water and extensive aquaculture industries are also located here. *Cochlodinium polykrikoides* (Dinophyceae) is one of the most prevalent harmful dinoflagellates and is responsible for fish kills in western Japan (Yuki and Yoshimatsu, 1989; KFCO, 2002), southern Korea (Kim, 1998) and China (Du *et al.,* 1993). A bloom of *C. polykrikoides* was reported for the first time from Yatsushiro Sea, Kumamoto Prefecture, in summer 1977, and was associated with a mortality of farmed fish. The bloom occurred again in 1978, causing massive damage valued at about 10 billion yen (Honda *et al.,* 1980). Since 1977, *C. polykrikoides* blooms have frequently occurred from early July to early September in the Yatsushiro Sea (KFCO, 2002). In the summer of 2000, a massive bloom of this organism caused heavy financial damage valued at about 40 billion yen; the greatest damage ever recorded by this organism in Japan. Although such harmful events occur frequently, little is known about the environmental conditions during the developing phase of the *C. polykrikoides* blooms. This paper describes the developmental process and the environmental characteristics of the *C. polykrikoides* bloom in 2000.

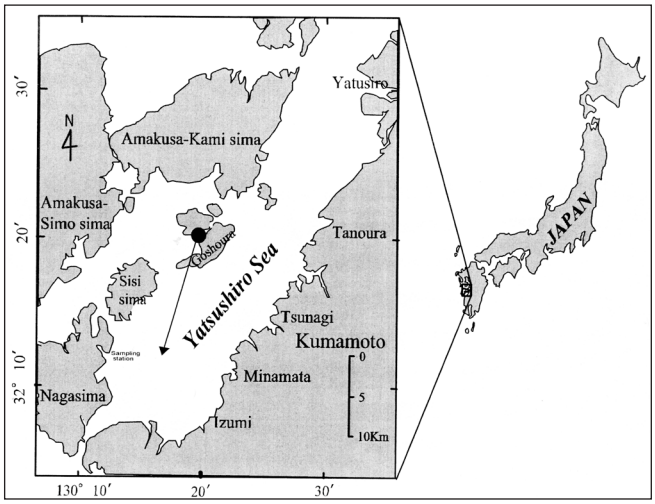

Figure 1 Map of Yatsushiro Sea showing the sampling station.

Materials and Methods

Field investigation was carried out at a fixed station (Fig. 1, water depth ca. 30 meters) during May–October 2000. Seawater samples were collected with a Niskin water sampler at weekly intervals at six different depths (0, 2, 5, 10, and 15 meters, and at 1 meter above the bottom). Water temperature and salinity were determined using a CTD system (YSI, model 85) at each sampling time (09:00–10:00). Seawater samples for nutrient analysis were filtered through GF/C membrane filters immediately after sampling, and stored at –20°C until analysis. Nutrients were analyzed using a Traacs 800 Autoanalyzer (Bran-Luebbe) according to the method of Strickland and Parsons (1972). Live cells of *C. polykrikoides* and other phytoplankton were counted one day after sampling because the samples had to be transported from the Yatsushiro Sea to our laboratory. *In situ* growth rates (division day^{-1}) of *C. polykrikoides* were estimated from changes in cell numbers according to Guillard's formula (Guillard, 1973). Precipitation data were obtained from the monthly weather reports for Yatsushiro City, Kumamoto Prefecture.

Results and Discussion

Development of a Cochlodinium polykrikoides *Bloom*

The massive bloom of *C. polykrikoides* that occurred in the Yatsushiro Sea in 2000 initially appeared in the eastern part of the sea, off Tsunagi, on 7 July, and then disappeared by 1 August. The bloom spread quickly over the whole sea, carried by current and tide, eventually covering more than 59 km^2. The bloom caused the mortality of about 2,618,000 cultured yellowtail, globefish and sea bream. The financial cost amounted to about 40 billion yen. The maximum cell density recorded was 28,250 cells/mL on 17 July in the central part of the sea (KFCO, 2001). Weekly temporal changes in cell densities and *in situ* growth rates (A) and the vertical distribution (B) of *C. polykrikoides* during the periods of observation at the fixed station are shown in Fig. 2. The cell densities are expressed as mean values for the water column. *Cochlodinium polykrikoides* were first observed at 0.002 cells/mL on 5 June. The cell density then reached a maximum of 700 cells/mL on 24 July and later decreased rapidly. The bloom appeared on 10 July and continued until 24 July during the period of our observa-

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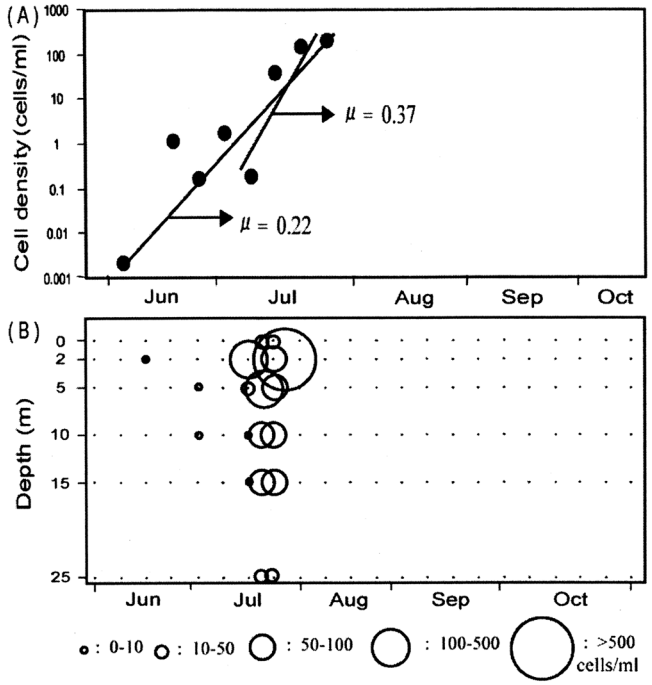

Figure 2 Temporal changes in mean cell density and growth rates (div. day^{-1}) (**A**), and vertical distribution (**B**) of *C. polykrikoides* in the water column during observation periods.

tion. Growth rates between 5 June and 24 July and between 3 and 24 July were calculated as 0.22 and 0.37 divisions day^{-1}, respectively (Fig. 2A). These growth rates were estimated without consideration of the effects of cell losses and/or gains such as biological and physical factors attributing to population growth. Nevertheless, the values are approximately in accord with the growth rates obtained in culture experiments under controlled conditions of light, temperature, salinity and nutrients. The optimal growth rates for *C. polykrikoides* cultures have ranged from 0.3–0.41 divisions day^{-1} under conditions of enriched nutrients, temperature of 20°–27.5°C, salinity of 30–36 psu, and a light level of 110 μmol m^{-2} s^{-1} (Kim *et al.,* in press). The most abundant cell concentrations were regularly measured at the depth of two meters (Fig. 2B).

Environmental Conditions Figure 3 shows the vertical distribution of water temperature, salinity, dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) during the observation period at the fixed station. Water temperature and salinity during the *C. polykrikoides* bloom ranged from 24.5°–26.6°C and from 32.0–33.0 psu, respectively. A pycnocline was steadily formed at near 10 meters depth during the developmental period but broke down rapidly with the subsequent decline of the bloom (Fig. 3A, B). Many blooms have occurred within a temperature range of 22.4°–30.1°C, and a salinity of 30.0–35.8 psu in the field (Margalef, 1961; Honda *et al.,* 1980; Du *et al.,* 1993; Morales-Blake and Hernandez-Becerril, 2001). Growth experiments in culture have revealed that optimal growth rates were obtained when temperatures and salinities ranged from 20°–27.5°C and 30–36 psu, respectively (Kim *et al.,*

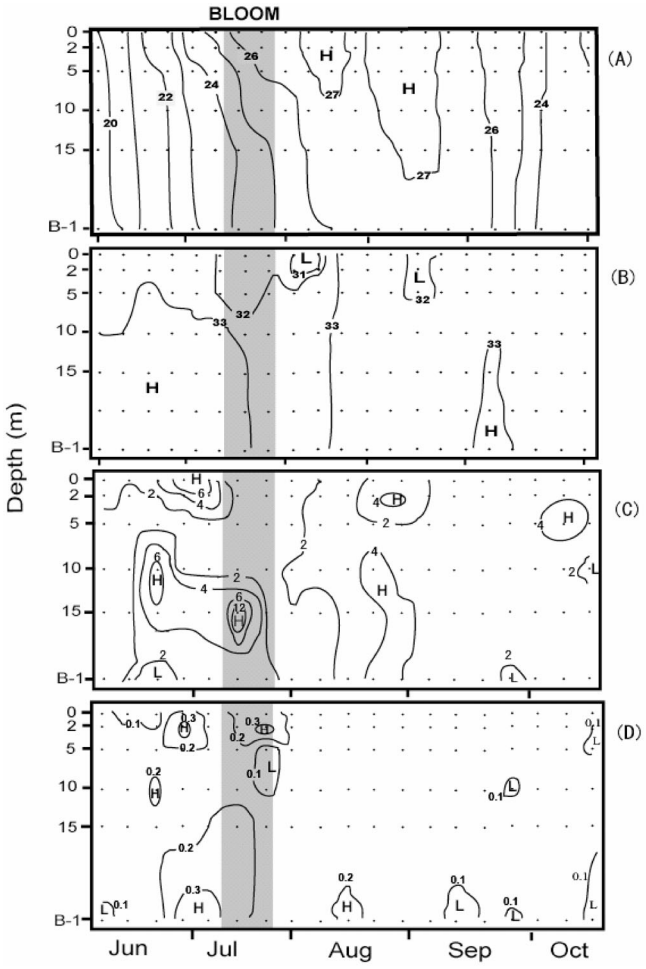

Figure 3 Vertical distribution of water temperature (**A**), salinity (**B**), dissolved inorganic nitrogen (**C**), and dissolved inorganic phosphorous (**D**). Dark bars denote the period of the *C. polykrikoides* bloom.

in press). These facts suggest that the bloom development in this organism is closely related to high water temperature and high salinity. During the observation period the concentrations of DIN and DIP ranged from 0.4–14.4 μM and from 0.01–0.38 μM, respectively. During the full bloom they ranged from 1.1–14.4 μM and from 0.06–0.34 μM, respectively, and were particularly high in the middle and bottom layers during the developmental period of the bloom (Fig. 3C,D). It is postulated that the elevated nutrients in the middle and bottom layers is supplied from sediment flux. Several bloom-forming dinoflagellates have the ability to take up and assimilate nutrients in the dark (Nakamura and Watanabe, 1983; Paasche *et al.,* 1984; Dixon and Holligan, 1989). *Cochlodinium polykrikoides* shows diurnal vertical migration at a speed of 1.4–3 meters/hour (Park *et al.,* 2001; Kim *et al.,* 2002). Therefore, we suggest that the *C. polykrikoides* bloom was formed and maintained by taking advantage of the nutrient-rich middle and bottom layer. Fig. 4 shows the relationship between bloom outbreaks and precipitation using 23 years of precipitation data from June–August of 1978–2000 in the Yatsushiro Sea area. When *C. polykrikoides* blooms occurred, the precipitation was lower than those years in

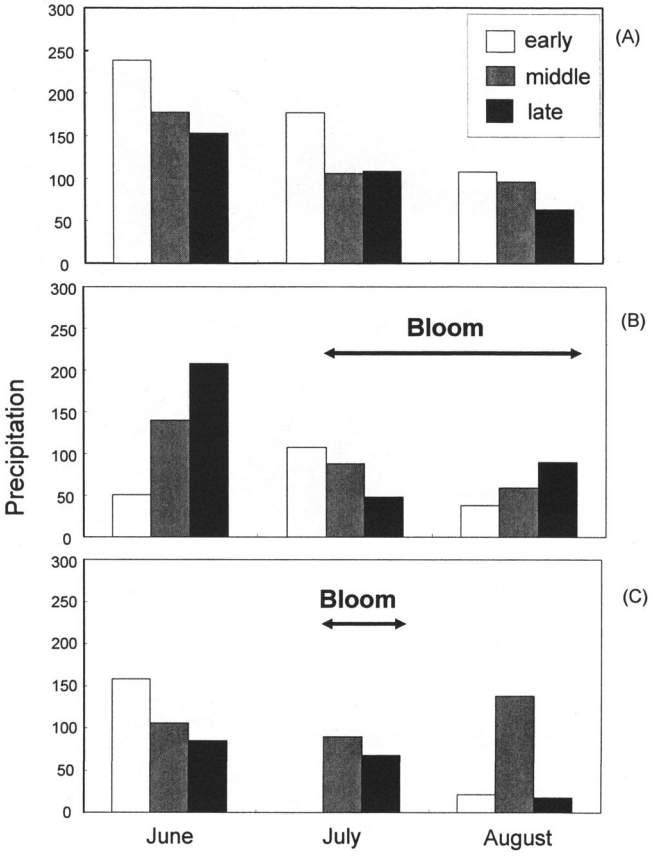

Figure 4 Relationship between bloom and precipitation from June to August 1978–2000 in the Yatsushiro Sea. **A** Non-bloom year, **B** Bloom year, **C** 2000.

which there were no blooms. In particular, the precipitation was extremely low in early July 2000. The precipitation data implies that *C. polykrikoides* blooms occur in drought years but not in rainy years. In addition, as a stenohaline species, *C. polykrikoides* is physiologically sensitive to changes in salinity (Kim *et al.,* in press). Furthermore, after the rains came on July 24, the bloom disappeared (M. Iwasaki, pers. comm.). The rainfall-lowered drop in the salinity of the seawater might be significant in the termination of blooms in the Yatsushiro Sea. Therefore, high salinities brought on by drought conditions during periods of high water temperature appear to be an essential condition for *C. polykrikoides* blooms.

Cochlodinium polykrikoides and Other Phytoplankton

The successional pattern of the most predominant phytoplankton, *Cochlodinium polykrikoides, Karenia mikimotoi, Chattonella antiqua, C. marina, Skeletonema costatum* and *Chaetoceros* spp., during the investigation period is shown in Fig. 5. In the initiation period of the *C. polykrikoides* bloom, all species were observed, but during the full *C. polykrikoides* bloom from 10–24 July, these species disappeared. After the decline of the bloom, *K. mikimotoi, Chattonella antiqua, C. marina, S. costatum,* and *Chaetoceros* spp. reappeared. In further experiments, it will be necessary to examine species competition, such as between *C. polykrikoides* and other flagellates or between *C.*

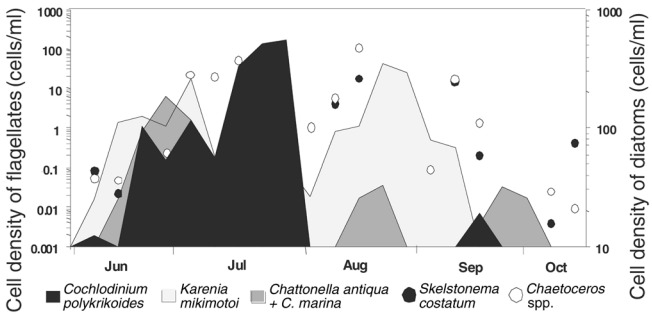

Figure 5 Temporal changes in predominant phytoplankton during the periods of observation. The cell density represents the mean value for the water column.

polykrikoides and diatoms, to understand some of the mechanisms for the development of *C. polykrikoides* blooms.

Acknowledgements

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HAB Distribution and Associations with Environmental Variables– Delaware Estuarine Coastal Bays, USA, 1998–2002

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Abstract

Fourteen HAB species known to produce biotoxins have been identified in the Delaware Inland Bays during routine monitoring between 1998 and 2002. To examine the association between a HAB species (*i.e.,* a dichotomous response variable) and continuous explanatory environmental variables, a logistic regression model was developed for selected HAB species. Based on data available at the time of the model development, there was a suggested association between *Pfiesteria piscicida* and Dissolved Kjeldahl Nitrogen; *Pfiesteria shumwayae* and Total Nitrogen, Total Dissolved Nitrogen, and Total Organic Nitrogen; *Karlodinium micrum* and Dissolved Organic Nitrogen and CBOD-5 day; and *Chattonella* (*C. subsala* and *C.* cf. *verruculosa*) and Total Nitrate/Nitrite, Dissolved Ammonium and Dissolved Inorganic Nitrogen.

Introduction

The Delaware Inland Bays, covering approximately 32 sq. mi., are comprised of Rehoboth Bay, Indian River/Bay and Little Assawoman Bay, with a narrow, shallow canal connecting the northern bays from the southern Little Assawoman Bay. The State of Delaware initiated the Delaware Inland Bays *Pfiesteria* Routine Surveillance Monitoring Program in May 1998 with project grant funds from the USEPA, Region III. In 2000, the program was expanded to include monitoring of *Karlodinium micrum.* In 2001, the State initiated surveillance monitoring of *Chattonella* (*C.* cf. *verruculosa* and *C. subsala*) following identification of this genus during an episodic event in 2000 [Delaware Dept. Natural Resources and Environmental Control (DDNREC) HAB Program database].

Methods and reporting criteria for HAB surveillance monitoring data are presented in the Program Metadata in the yearly HAB work plan available from the DDNREC, Division of Water Resources, and Environmental Laboratory Section and in Humphries (2003b). Results from 1998–2002 are entered and archived as the HAB Program in the State Laboratory Information Management System (LIMS).

To date, 14 HAB species known to produce biotoxins have been identified in the Delaware Inland Bays, including *Chattonella subsalsa, C.* cf. *verruculosa, Dinophysis norvegica, Fibrocapsa japonica, Heterosigma akashiwo* (formerly *H. carterae*), *Karlodinium micrum, Microcystis aeruginosa, M. incerta, Pfiesteria piscicida, Pfiesteria shumwayae, Prorocentrum micans, Prorocentrum minimum, Pseudo-nitzschia pungens,* and *Pseudo-nitzschia seriata.* This report evaluates the association between the four most frequently identified HABs (*Pfiesteria piscicida, P. shumwayae, Karlodinium micrum,* and *Chattonella* spp.) and environmental variables monitored.

Materials and Methods

The data set consists of the four previously mentioned HAB species, plus physical, chemical and biological variables. The HAB species data are dichotomous (present or not detected) and the 45 environmental variables, which were either measured or calculated, are continuous. All variables monitored were analyzed from monthly or bi-monthly collections of surface or whole column water samples collected at fixed stations during the growing season (May–October) from 1998–2002. Physical variables monitored included surface and bottom water temperature, salinity, dissolved oxygen (DO) and specific conductivity; surface pH; wind direction and speed; percentage of cloud cover; water odor and color; and Secchi and station depth. Chemical variables measured included ammonium (total, dissolved), nitrate/nitrite (total, dissolved), Kjeldahl nitrogen (total, dissolved), dissolved urea, phosphorus (total, inorganic), soluble reactive silicate (Si), carbon (total, dissolved), CBOD-5, and total suspended solids (TSS). Biological variables included chlorophyll *a* and phaeophytin. The 17 calculated variables were: Particulate Carbon [= Total Organic Carbon (TOC) – Dissolved Organic Carbon (DOC)], Particulate Nitrogen [PN = Total Nitrogen (TN) – Total Dissolved Nitrogen (TDN)], Particulate Phosphorus [= Total Phosphorus (TP) – Total Dissolved Phosphorus (TDP)], Dissolved Inorganic Nitrogen [DIN = Dissolved Ammonium (DNH3) + Dissolved NitrateNitrite Nitrogen (DNO3NO2)], Dissolved Organic Nitrogen [DON = Dissolved Kjeldahl Nitrogen (DKN) – DNH3], Total Dissolved Nitrogen [TDN = DKN + DNO3NO2], Total Nitrogen [= Total Kjeldahl Nitrogen (TKN) + Total Nitrate/Nitrite Nitrogen (TNO3NO2)], Total Organic Nitrogen [TON = TKN – Total Ammonium Nitrogen (TNH3)], Dissolved Organic Phosphorus [DOP = TDP – Dissolved Ortho-Phosphate], DIN/DIP [DIN/Dissolved Inorganic Phosphorus], DOC/DON, DON/DIN, DON/DOP, Si/DIN, TN/Si, TN/TP, and "Secchi Depth/Water Column Depth." Molecular DNA probe analyses were used to identify *Pfiesteria piscicida, P. shumwayae* (Rublee *et al.,* 1999; Glasgow and Burkholder 2000; Oldach *et al.,* 2000), *Karlodinium micrum,* and *Chattonella* spp. (D. Oldach, University of Maryland, Baltimore).

The purpose of this modeling effort was to explore relationships among HAB species, and between HAB species

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and a suite of explanatory variables. The modeling effort proceeded in three phases. The first was to investigate the association between selected HAB species. This was accomplished with a series of 2 × 2 chi-square tests of independence. A small *P*-value indicates a high level of dependence (association). A *P*-value above 0.05 indicates that the association may be minimal. The second phase was an initial screening of the 45 continuous explanatory variables against each of the four HAB species using a logistic regression model. This resulted in 45 individual logistic regression models for each species. The third phase was the construction of models based upon results from the initial screening using multiple explanatory variables. Model significance is indicated by *P*-values and deviance. Deviance is similar to R-square in multiple regression; a high value indicates a strong association.

A multiple comparison problem occurs when determining significance for a suite of single-factor logistic regression screening models. The consequence is that models or variables in a model may appear to be statistically significant when they are not. This problem was addressed with the Dunn-Sidak method (a modification of the well-known Bonferroni method). This method established an experimentwise inclusion criterion of 0.05 for variables to be admitted into the multi-factor logistic regression model. It also provided conservative error rates on the tests of significance for the individual models because many of the explanatory variables are not independent (Sokal and Rohlf 1995), as shown with the 17 calculated variables. Since the nature of this study is exploratory, the levels of significance stated in the results should be considered as guidelines. Subsequent model development utilizing additional data may provide additional validity to these results.

Results

The observation of *Pfiesteria piscicida* was related to that of *P. shumwayae* for 2000 and 2001 as determined by the 2 × 2 chi-square test of independence; *P*-value = <0.0001 and *P*-value = 0.0001, respectively. The observation of *P. piscicida* was related to that of *Karlodinium micrum* for 2000 and 2001, again based upon a 2 × 2 chi-square test of independence. The strongest association was recorded in 2001 (*P*-value = 0.0001 compared to *P*-value of 0.0524 in 2000). The observation of *P. shumwayae* was not related to the observation of *K. micrum* for either 2000 or 2001; *P*-value = 0.7013 and *P*-value = 0.3660, respectively.

The composite 1999–2001 data set was used in the logistic regression model for *Pfiesteria piscicida* and *P. shumwayae*. *P. shumwayae* showed a significant single-factor relationship with more nutrient analytes than did *P. piscicida*.

Based on the single-factor logistic model screening, the initial model for *P. shumwayae* was formulated using the following explanatory variables: TDN, DKN, TN, TON, DOC/DON, DON, DON/DIN, DOP, Urea, TDP, TKN, DOC, phaeophytin, and surface and bottom temperature (Humphries 2003a). The final model (*P*-value = <0.0001, df = 225, 43.5% deviance) included the following variables in decreasing order of significance: TDN (*P*-value = <0.0001), TON (*P*-value = <0.0001), TN (*P*-value = 0.0027), phaeophytin (*P*-value = 0.0027), bottom water temperature (*P*-value = 0.0111), and surface water temperature (*P*-value = 0.0261).

The initial model for *P. piscicida* was formulated using the following explanatory variables: TDN, DKN, DOC/DON, DON, surface and bottom salinity, and station depth. The final model (*P*-value = < 0.0001, df = 261, 20.8 % deviance) included the following variables each of equal significance (*P*-value = <0.0001): DKN and station depth.

The association between *Karlodinium micrum* and environmental variables was formulated using the composite 2000–2001 data set. All variables having a *P*-value < 0.10 in the screening logistic regression model were included in the multi-factor logistic regression model. They were: DON, DON/DIN, DOP, Urea, CBOD-5, chlorophyll *a*, TP, PN, and TNH3. The final model (*P*-value = 0.0042, df = 65, 14.7% deviance) included the following variables in decreasing order of significance: DON/DIN (*P*-value = 0.0217), CBOD-5 day, and DON (*P*-value = 0.0415).

The association between *Chattonella* spp. and environmental variables was formulated using only the 2001 data. Thus, the model may not be as strong as if multiple years of data or data on a specific species were available. All variables having a *P*-value < 0.10 in the single-factor logistic regression were formulated in the multi-factor logistic regression model, and included the following: DNH3, TN03N02, DIN, DN03NO2, DIN/DIP, Si/DIN, DOP, and Secchi and station depth. The final model (*P*-value = < 0.0001, df = 57, 35.4% deviance) included the following variables in decreasing order of significance: TN03NO2 (*P*-value = 0.0001), DNH3, DIN, and Secchi depth (*P*-value = 0.0160).

Discussion

The models presented are based on available data and represent the results of an initial screening technique to examine the association between a HAB species occurrence and environmental variables measured or calculated at the time of the documented occurrence of the species. The multi-factor logistic regression model has been demonstrated as a useful tool to examine organismic-environmental associations in a field application. Based on the logistic regression model developed for each *Pfiesteria* species using the composite 1999–2001 data set, *Pfiesteria piscicida* was associated with only the nutrient DKN, whereas *P. shumwayae* was associated with TN, TDN, and TON. Of the two models, the model is stronger for *P. shumwayae* based on a 43.5% model deviance as compared to 20.7% for *P. piscicida*.

The model for *Karlodinium micrum* suggests an association with DON, DON/DIN, and CBOD-5 day. The percent deviance explained by the model was 14%, which is low. The strength of the model may change as additional data become available. The model for the combined *Chattonella* species suggests an association with TN03N02, DNH3 and

DIN. The percent deviance explained by the model was 35%; however it is based on only one year of data, and is further limited by combining the two Chattonella species, which do not always co-occur (DDNREC, LIMS).

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Dinoflagellate Blooms Related to Coastal Upwelling Plumes Off Portugal

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Abstract

The different hydrodynamic and ecological conditions that favour the development of *Gymnodinium catenatum* and *Lingulodinium polyedrum* blooms along the upwelling coast of Portugal are discussed based on previous results, on SST satellite images and on resting cyst distributions. Results indicate that each species benefits from particular ecological niches associated with upwelling plumes. *L. polyedrum* explores warm stratified waters adjoined by upwelling nutrient-rich plumes, and *G. catenatum* benefits from physical mechanisms favouring its accumulation and leading to a "pelagic seed bank." In these two species, cysts seem to play a different role in bloom dynamics.

Introduction

Gymnodinium catenatum and *Lingulodinium polyedrum* are considered as "Upwelling Relaxation Taxa" (Smayda, 2002) and both species have developed blooms along the upwelling coast of Portugal. Blooms of *G. catenatum* were regularly observed between 1985 and 1995, covering wide areas of the coast. Extensive and massive blooms of *G. catenatum* were recorded in autumn (*e.g.,* 1985 and 1994) and were preceded, in summer, by the detection of the motile stage in central Portugal, when the species was apparently accumulated in association with upwelling plumes (Moita *et al.,* 1998; 2003). Blooms of *L. polyedrum* are apparently more sporadic and geographically limited, with the few records involving water discoloration. In September 1996, a *L. polyedrum* bloom was recorded south of Lisbon in Setúbal Bay (Amorim *et al.,* 2001b). This bloom developed in stratified waters inside the bay, while wind data suggested active upwelling was occurring in the area. Here we review some of these previous results and present new data on SST satellite images and on the distribution of resting cysts in order to investigate differences on the hydrodynamic and ecological conditions that may favour the development of *G. catenatum* and *L. polyedrum* blooms.

Materials and Methods

Surface sediments were collected from a variety of coastal environments using a simple sucking device (Dale and Dale, 2002). Three sites were sampled for time-series studies of cyst assemblages: on the NW (Aveiro), SW (Sines) and S (east of Cape S. Vicente). The site located on the SW coast was sampled every two months for more than three years, starting in February 1996, while the others were sampled seasonally, for one year and a half, starting in 1998. Sediment samples were kept cool and in the dark until treatment according to Amorim and Dale (1998). Samples were examined for both empty cysts and cysts with cell contents. A total of at least 200 cysts were counted. Results are expressed as % values of the whole cyst assemblage. Infrared images for the study region were derived from the Advanced Very High Resolution Radiometer (AVHRR) carried on the NOAA polar orbiting satellites. Global area coverage data were obtained from the NOAA Satellite Active Archive. The brightness temperature from AVHRR channel 4 (11.5–12.5 μm) was used as an estimate of the sea surface temperature (SST). The grey shading was individually selected to enhance the thermal structures of interest. Darker shades correspond to higher SST values.

Results and Discussion

Cysts of *L. polyedrum* were ubiquitous along the coast of Portugal, with a main distribution centre close to Cape S.

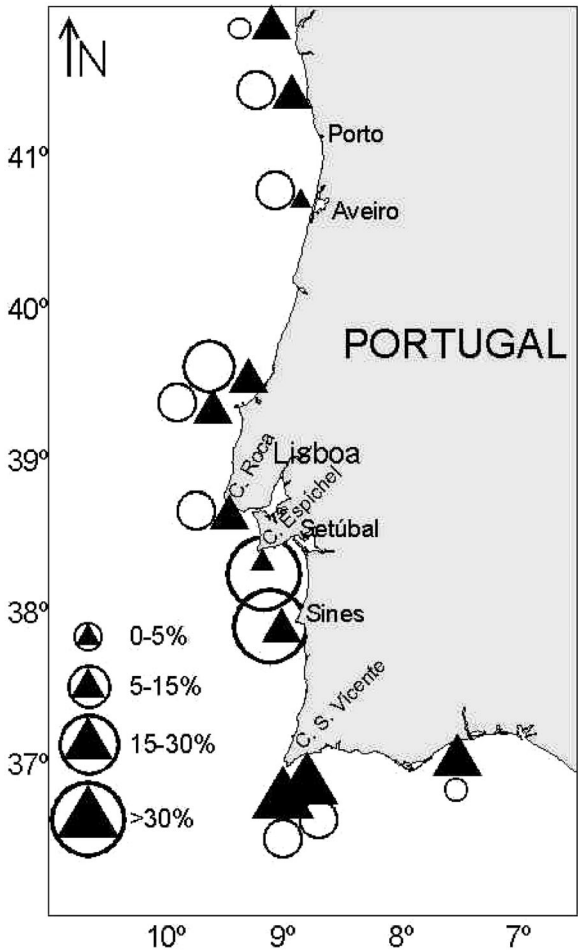

Figure 1 *L. polyedrum* (▲) and *G. catenatum* (◯) cyst distribution along the coast of Portugal. Results are expressed as % of whole cyst assemblage, and for each site, maximum values are presented.

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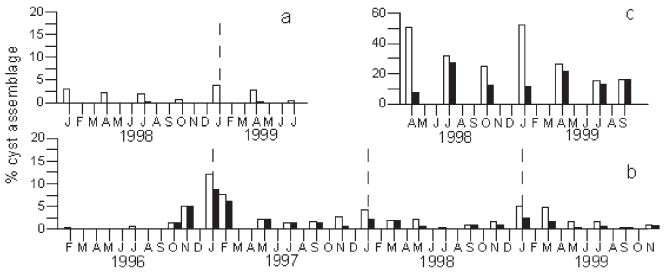

Figure 2 Seasonal variation of the relative abundance of *L. polyedrum* cysts on the (**a**) NW, (**b**) SW and (**c**) S coasts. The empty columns refer to total cysts (empty + cell contents) and the full columns to cysts with cell contents. Note: scale for the S coast is different.

Vicente (Fig. 1). At this site, time-series studies indicate that *L. polyedrum* consistently dominates cyst assemblages, while at the SW coast it never exceeds 12% and at the NW coast the maximum recorded value was 4% (Figs. 2a–c). In turn, the distribution of cysts of *G. catenatum* showed a main distribution centre in the central region of the W coast (Fig. 1) and reflected the distribution pattern of *G. catenatum* blooms in autumn 1994 and 1995 (Moita *et al.,* 1998). On the SW coast, cyst assemblages showed an increase in *L. polyedrum* cysts with cell contents from October 1996 to January 1997 (Fig. 2b). These observations have previously been linked to the *L. polyedrum* bloom recorded farther north, in Setúbal Bay, during September 1996, and showed that there is ca. 1 month time-lag between the presence of cysts in the water column and the increase in newly formed cysts in sediments (Amorim *et al.,* 2001b). Retrospective analysis of a sequence of SST satellite images shows that at the time of the bloom, the upwelling plumes rooted at capes Roca/Espichel, which normally extend southward or westward, were unusually displaced shoreward into Setúbal Bay, probably due to the influence of the offshore mesoscale circulation (Fig. 3a). The bloom developed in warmer waters inside the bay (Fig. 3a, arrow), between the offshore upwelling plume and an upwelling front present inshore (Amorim *et al.,* 2001b).

In the Cape S. Vincente area, cysts of *L. polyedrum* with cell contents reached their highest proportions in July 1998 (Fig. 2c). Based on the time-lag observed in Setúbal Bay between detection of the bloom and detection of cysts in sediments, SST satellite images one month prior to sampling were analysed. Results indicate that surface ocean circulation at the cape was characterised by an eastward advection of cold upwelled waters extending over the shelf break and slope of the southern coast. This plume was separated from shore by a narrow band of warm waters (Fig. 3b, arrow). Satellite imagery showed the westward progression of this warmer counterflow along the S coast reaching Cape S. Vicente. This warm water band persisted for ca. 2 weeks in the area of the cape (Fig. 3b). The interaction of these two water bodies, the upwelling plume and the inshore warmer waters, may have favoured the development of *L. polyedrum* blooms, as the cyst record suggests. According to Relvas and Barton (2002), during summer, the W coast in the vicinity of Cape S. Vicente is characterised by an almost permanent upwelling regime, under northerly winds, and an associated equatorward flow of cool waters that preferentially turn the cape flowing eastwards along the S shelf break. In turn, the S coast is characterised by the presence of a warm coastal countercurrent that flows westwards over the continental shelf. The extent of its westward progression depends on the intensity and persistence of the W coast upwelling and on occasional S coast upwelling induced by local westerly winds. The particular conditions recorded in June 1998 in the area of the cape (Fig. 3b), *i.e.,* the interaction during two weeks of the two water bodies, are an annual recurrent feature during the upwelling season (Relvas and Barton, 2002). The high background levels of *L. polyedrum* cysts in sediments indicate this species thrives in the area, possibly in response to those conditions. Both the cyst record at Cape S. Vicente and the 1996 bloom in Setúbal Bay suggest that *L. polyedrum* explores an ecological niche associated with the interaction of warm stratified waters compressed by adjoining upwelling nutrient-enriched plumes. However, in Setúbal Bay these conditions are presumably less frequent since there is no clear cyst signal.

Present day cyst distributions have allowed the identification of marine environmental signals. High proportions of *L. polyedrum* have been identified as indicating an increase in nutrient input, either natural (upwelling) or from cultural eutrophication (Dale, 1996). The Cape S. Vicente region is an exposed site, free from any anthropogenic sources of nutrients. Thus, the very high relative abundance values found for cysts of *L. polyedrum* here are interpreted as an upwelling signal related to the peculiarities of this process at the cape. This area may be compared to other upwelling areas in the world, namely S California and NW Africa, where cysts of *L. polyedrum* dominate assemblages on the shelf (Dale and Dale, 2002). In all these cases, waters over the shelf are somewhat enriched by nutrients from upwelling waters present offshore (Dale and Dale, 2002).

Upwelling plumes also seem to play an important role in the dynamics of *G. catenatum* blooms in central Portugal. The combined analysis of phytoplankton data and sequences of satellite SST images showed that the massive blooms of *G. catenatum* that occurred off W Iberia during autumns of 1985 and 1994 were preceded, in summer, by the detection of the motile stage in association with capes Roca and Carvoeiro plumes (Moita *et al.,* 2003). These authors showed that the species was apparently accumulated along the eastward side of the Cape Roca upwelling plume extending southward, within warmer waters, by a possible weaker northward flow. A similar situation was described to occur along the leeward side of those plumes extending westwards where this species was observed to accumulate near the coast, close to the core of the upwelling plume without being advected away. The accumulation of *G. catenatum* by the physical mechanisms described reinforce the idea that the species may reach the threshold concentrations for bloom initiation from an innoculum in the water column

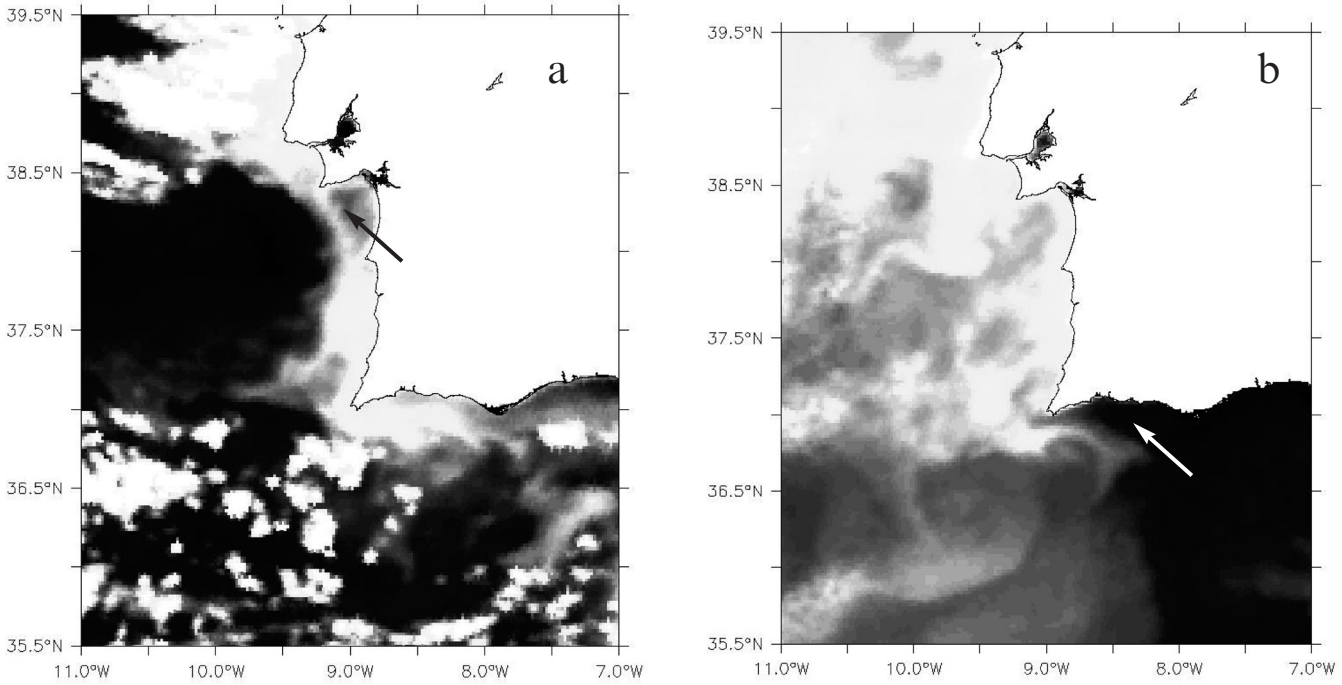

Figure 3 Thermal infrared NOAA–AVHRR images of **a** 3 Sept 1996 showing the upwelling plumes rooted at capes Roca and Espichel being unusually displaced shorewards, limiting warmer waters inside Setúbal Bay (arrow), and **b** 23 June 1998, showing the eastward advection of the Cape S. Vicente upwelling plume and presence of an inshore warm water band in the S coast, reaching the cape (arrow).

and support the hypothesis that blooms may originate from a "pelagic seed bank" (Dale and Amorim, 2000; Moita and Amorim, 2002; Smayda, 2002).

The cyst stage of *G. catenatum* and *L. polyedrum* are likely to also play a different role in bloom dynamics. With the exception of the NW coast, *L. polyedrum* cysts were always recorded in high proportions with apparently viable cell contents (Figs. 2a–c), suggesting the build up of a cyst bed, while *G. catenatum* cysts were mainly empty (95–100% of total *G. catenatum* cysts) (Amorim *et al.,* 2001a). These differences may be explained based on the different mandatory dormancy periods described for these two species and on the physiology of the cysts. For *L. polyedrum,* the dormancy period is believed to be of several months and cyst germination dependent on environmental conditioning (*e.g.,* anoxia) (Lewis and Hallet, 1997), while cysts of *G. catenatum* have a very short dormancy period ca. 12 days and do not require any environmental conditioning prior to germination. As previous work suggests, bloom dynamics of *G. catenatum* do not depend on the build-up of a cyst seed bed, while results presented here on *L. polyedrum* suggest that, for this species, cysts may be a key stage on the seeding and timing of blooms (Dale andAmorim, 2000; Amorim *et al.,* 2001a,b; Moita andAmorim, 2002).

Acknowledgements

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The Relationship of *Alexandrium fundyense* to the Temporal and Spatial Pattern in Phytoplankton Community Structure Within the Bay of Fundy, Eastern Canada

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Abstract

The spatial and temporal variation in abundance of *Alexandrium fundyense* in relation to the spatial and temporal variation in phytoplankton community structure at a series of monitoring stations within the Bay of Fundy is examined through a multi-dimensional scaling ordination analysis conducted on data collected in 1991. The results indicate a strong seasonality in both the abundance of *A. fundyense* and in the community structure. While *A. fundyense* is present sometimes at all sampling stations, it is abundant at only a few and is most abundant during July. The distribution of *A. fundyense* may therefore be related to the seasonality in the phytoplankton community or its correlates, such as the environmental conditions. The spatial distribution of *A. fundyense* appears to be independent of the phytoplankton community structure.

Introduction

Alexandrium fundyense, the organism responsible for paralytic shellfish poisoning (PSP), blooms annually in the Bay of Fundy, generally during the months of May–August. Although considerable effort has been made over the years to identify the factors and processes controlling the bloom dynamics of *A. fundyense* (*e.g.,* Taylor 2000), few studies have examined how *A. fundyense* varies in relation to the structure of the overall phytoplankton community. Such an approach may provide some additional insights into the population dynamics, and environmental factors and processes influencing *A. fundyense* distribution and abundance. In turn, this may help to develop predictions of occurrence and toxicity of *A. fundyense,* and ultimately, help improve management of harmful algal blooms (HABs).

The present study describes the temporal and spatial variation in the concentration of *A. fundyense* and the phytoplankton community structure in the Bay of Fundy and the temporal and spatial association between the two.

Materials and Methods

A phytoplankton-monitoring program has been maintained in the Bay of Fundy since 1987 (Martin *et al.,* 1995, 1999, 2001). As part of this program, water samples were collected from the near-surface at weekly to monthly intervals from 4 stations (3,15,16 and 17) throughout 1987 to 2000 and from an additional 8 stations during 1991 (12 stations in all). The stations were located in the Bay of Fundy, eastern Canada (Fig. 1). Phytoplankton cells have been identified and enumerated microscopically following settlement of 50 mL of each water sample using the Utermöhl technique. *A. fundyense* identification has been confirmed by scanning electron microscopy.

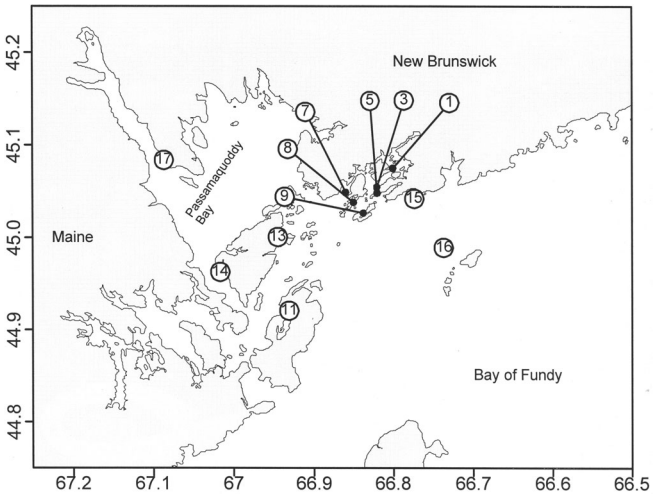

Figure 1 Location of sampling stations.

The above dataset was used to generate time series of *A. fundyense* and to examine the relationship of *A. fundyense* to the overall structure of the phytoplankton community in the Bay of Fundy. For the latter, we used the spatially more extensive 1991 data. We summarized the phytoplankton community structure with a multi-dimensional scaling (MDS) ordination of a Bray-Curtis similarity matrix that was generated from the log10 transformed cell counts per liter of each phytoplankton species collected in each sample. The MASS package (version 7.1-11) for R (version 1.8.0) was used to conduct the MDS analyses (Venables and Ripley, 2002).

Results and Discussion

The time series of *A. fundyense* indicates it occurred in each year from 1987 through 2000 at the four long-term monitoring stations (3, 15, 16 and 17) in the Bay of Fundy (Fig. 2). The cell counts ranged from 20 to 1.6×10^5 cells $\cdot$ L^{-1}, and each year the cell counts usually exceeded the concentration (200 cells $\cdot$ L^{-1}) needed to generate shellfish PSP toxin levels that are above the regulatory limits. Over the length of the time series, the annual *A. fundyense* bloom occurred anytime between May and August but generally occurred in July. In 1991, *A. fundyense* occurred predominantly during the month of July with notable concentrations in June and September (Fig. 3).

The spatial distribution of *A. fundyense* is indicated by the 1991 data (Fig. 1, 4). The highest concentrations of *A. fundyense* were found offshore (station 16). Somewhat

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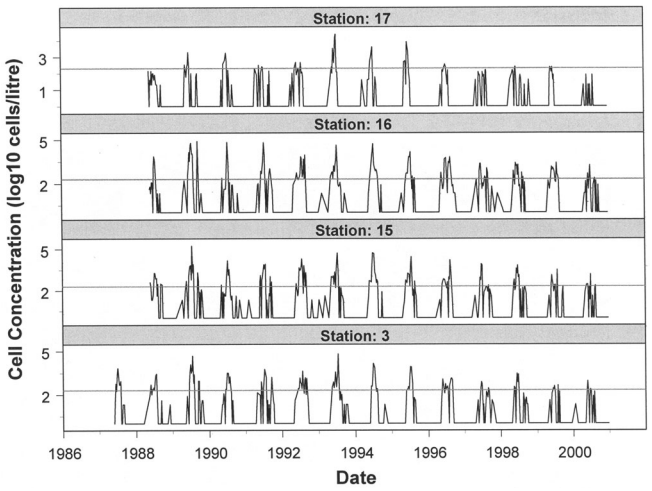

Figure 2 Time series of *A. fundyense* abundance at monitoring stations 3, 15, 16 and 17. The horizontal reference lines indicate cell concentrations of 200 cells L^{-1}.

lower, but still relatively high concentrations occurred in the Letang Inlet area (*e.g.,* stations 3,5,7,8,9 and 15). The lowest concentrations occurred in the inshore estuarine location (station 17). Low concentrations also occurred at the Deer Island (stations 13 and 14), Campobello Island (station 11) and Letang Estuary (station 1) sampling locations. This offshore to inshore spatial gradient in concentration is also reflected in the 1987–2000 time series (Fig. 2) and is consistent with the offshore survey results of Martin and White (1988).

The phytoplankton community in the Bay of Fundy is dominated by diatoms and dinoflagellates. The dominant species are listed in Table 1. The MDS ordination analysis of the 1991 data indicates three major patterns in the phytoplankton community structure. One pattern is that in any specific month, the phytoplankton community forms only a single cluster (Fig. 3) which suggests the stations do not have radically different phytoplankton communities. The second pattern relates to the variation in the tightness of the above clusters over months (Fig. 3) and suggests changing spatial similarity, with the similarity being greatest during April, May and June. The third pattern is indicated by the circular scatter of dots within each station (Fig. 4) and reflects the seasonality in the phytoplankton community structure.

The results indicate that in 1991 *A. fundyense* was present from April through October (Fig. 3) with the largest concentrations occurring during June, July and September (Fig. 3). Although *Alexandrium* was found throughout the sample domain it tended to be most abundant in the eastern portion (Fig. 1, 4) with the exception of station 1. *Alexandrium* occurred during months of relatively low and high degrees of phytoplankton community spatial similarity (Fig. 3). Peak *Alexandrium* abundance occurred during July, a month of relatively low spatial similarity (Fig. 3). Although in 1991 *Alexandrium* was most abundant in a subset of the sample stations, the community structure at these stations was not distinctly different from that at the other stations (Fig. 4). This suggests that the temporal distribution of *A. fundyense* may be related to some other factors that vary both seasonally and spatially.

It is well known that phytoplankton (including HABs)

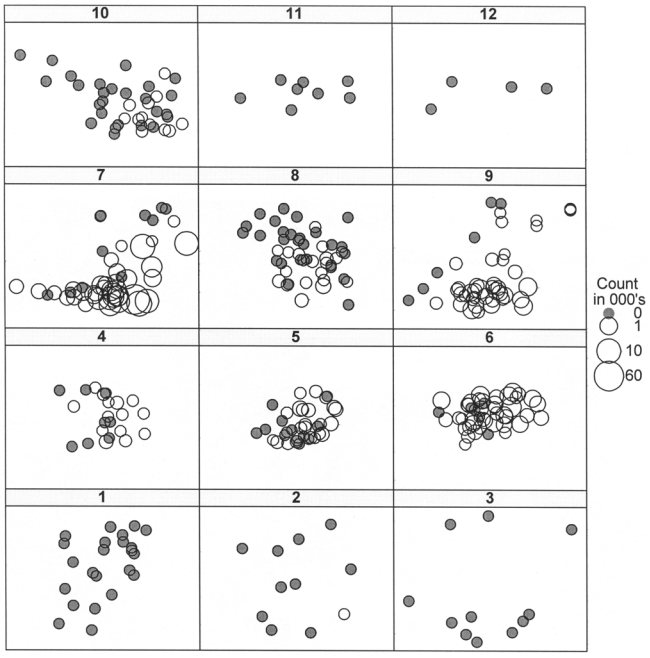

Figure 3 MDS ordination of 1991 phytoplankton samples grouped and centered by month (Jan = 1, Feb = 2, etc.). Each symbol represents one sample. The closer the symbols are to each other, the greater the similarity of the samples. The size of each symbol indicates *Alexandrium* abundance.

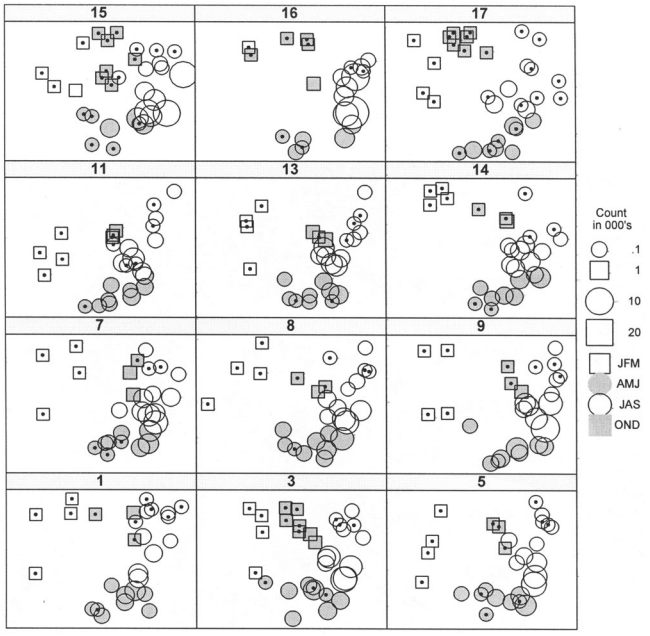

Figure 4 MDS ordination of the 1991 phytoplankton samples grouped by sampling station (1 through 17). Symbol size is proportional to *Alexandrium* abundance. Dots within symbols represent the absence of *Alexandrium.* The shape and shading of symbols represent seasons (J, F, M = Jan, Feb, Mar, etc.).

Table 1 Dominant phytoplankton species (>5000 cells · L^{-1} in a sample) detected in the study area. Potentially harmful species as identified by Martin *et al.* (1999) are indicated by bold type.

Diatoms		Dinoflagellates
Asterionellopsis glacialis	*Guinardia striata*	*Alexandrium fundyense*
Cerataulina pelagica	*Lauderia annulata*	*Ceratium lineatum*
Chaetoceros compressus	*Leptocylindrus danicus*	*Ceratium minutum*
Chaetoceros debilis	*Leptocylindrus minimus*	*Gonyaulax digitale*
Chaetoceros furcellatus	***Pseudo-nitzschia delicatissima* group**	*Gonyaulax spinifera*
Chaetoceros simplex	***Pseudo-nitzschia seriata* group**	***Gyrodinium aureolum***
Chaetoceros socialis	*Rhizosolenia* sp.	*Gyrodinium* spp.
Chaetoceros spp.	***Skeletonema costatum***	*Heterocapsa triquetra*
Cylindrotheca closterium	*Thalassionema nitzschioides*	***Prorocentrum minimum***
Dactyliosolen fragilissimus	*Thalassiosira angulata*	*Protoperidinium* spp.
Detonula confervacea	*Thalassiosira gravida*	*Scrippsiella trochoidea*
Ditylum brightwellii	*Thalassiosira nordenskioeldii*	
Eucampia zodiacus	*Thalassiosira rotula*	
Guinardia delicatula	*Thalassiosira* spp.	
Guinardia flaccida		

exhibit seasonal and interannual variability. Analyses of trends and patterns in specific species in combination with community and environmental structure may help advance understanding of the causes of this variability. We therefore intend to augment the above analyses by examining additional years of data, searching for associations between the phytoplankton and environmental parameters that have been collected as part of the monitoring efforts. We also hope to search for associations between time series of A. fundyense and PSP shellfish toxicity.

Acknowledgements

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Zooplankton Grazing During *Alexandrium* Blooms in the Gulf of Maine

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Abstract

Zooplankton grazing during natural blooms of *Alexandrium* spp. in the Gulf of Maine was non-selective. *Alexandrium* spp. and dominant microflagellates were each ingested in proportion to their availability.

Introduction

Some laboratory studies have suggested that toxic dinoflagellates repel zooplankton grazers (Turner and Tester, 1997). However, grazing has rarely been examined during natural blooms of toxic and non-toxic phytoplankton. During the Gulf of Maine ECOHAB program we examined grazing on *Alexandrium* spp. compared to other species.

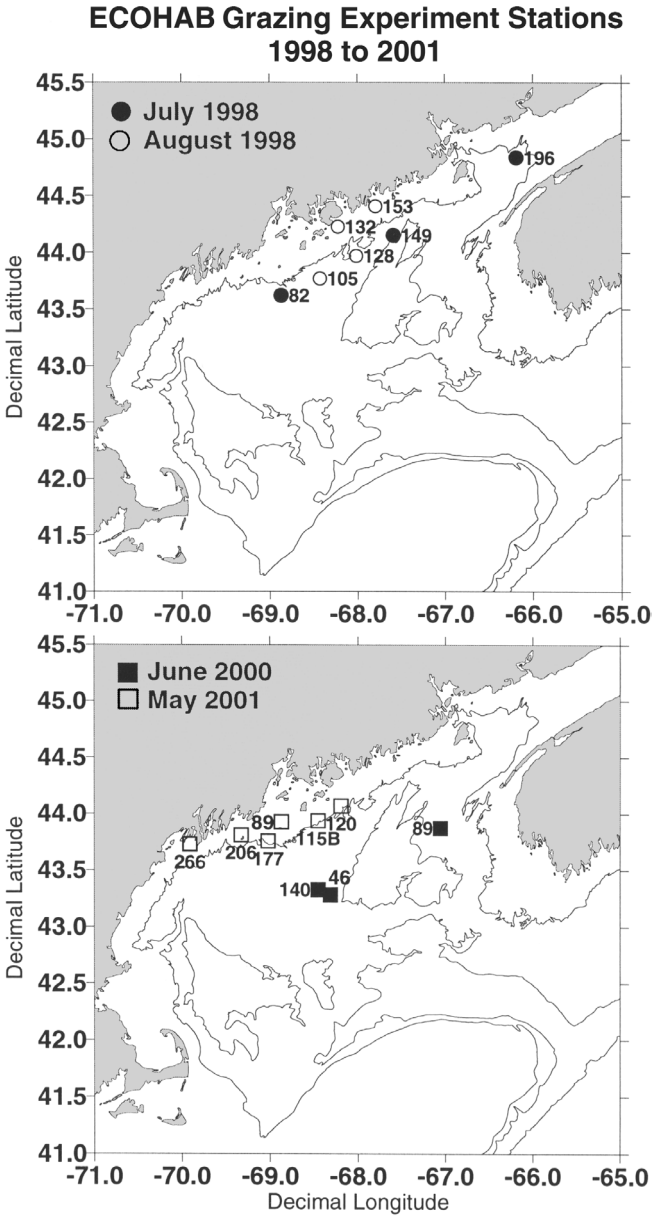

Figure 1 Stations where grazing experiments were conducted on cruises in July 1998, August 1998, June 2000, and May 2001.

Materials and Methods

Grazing was investigated by shipboard experiments during natural blooms of *Alexandrium* spp. in the offshore Gulf of Maine (Fig. 1) in spring and/or summer of 1998, 2000, and 2001. Several species of copepods, marine cladocerans and appendicularians (Fig. 2) were allowed to graze upon natural phytoplankton assemblages at natural abundances, at ambient temperatures (14–17°C) for incubations of 18–24 hours. Grazing for individual zooplankton taxa (mostly copepods) was measured by quantitative microscopic analyses of disappearance of *Alexandrium* spp. and all other cells in experimental, compared to initial and control suspensions, using the formulae of Frost (1972).

Results and Discussion

Even during *Alexandrium* "blooms," this dinoflagellate was a minor component of overall phytoplankton assemblages, present at stations where grazing experiments were con-

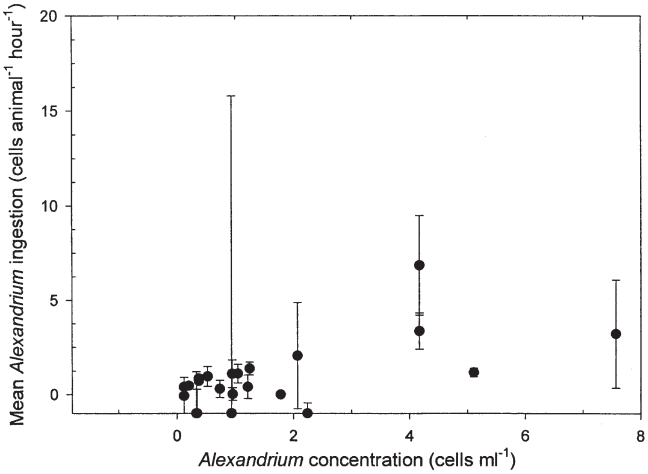

Figure 2 Ingestion rates (cells animal^{-1} hour^{-1}) of *Alexandrium* spp. versus *Alexandrium* concentrations (cells mL^{-1}) in surface waters for grazing experiments at stations shown in Fig. 1. Data points are means of 3 replicates, and vertical lines are + SD. Grazers were the most abundant zooplankters caught in 333 µm mesh surface net tows at each station, either adult females of the copepods *Centropages typicus* or *Acartia hudsonica,* copepodites of *Calanus finmarchicus,* the marine cladocerans *Evadne nordmani* or *Podon polyphemoides,* or the appendicularian *Oikopleura dioica.* In cases of calculated negative grazing, caused by counts of *Alexandrium* in grazed replicates that were higher than in the control (effectively where the number of *Alexandrium* cells removed by grazing was lower than the minimum counting precision of cells), means are plotted as –1.0 but SD values are plotted as calculated.

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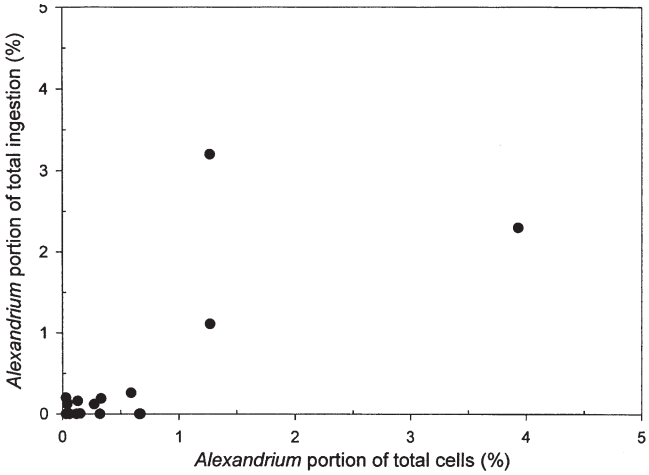

Figure 3 Percentage of total grazing comprised by grazing on *Alexandrium* compared to percentage of total phytoplankton abundance comprised by *Alexandrium.*

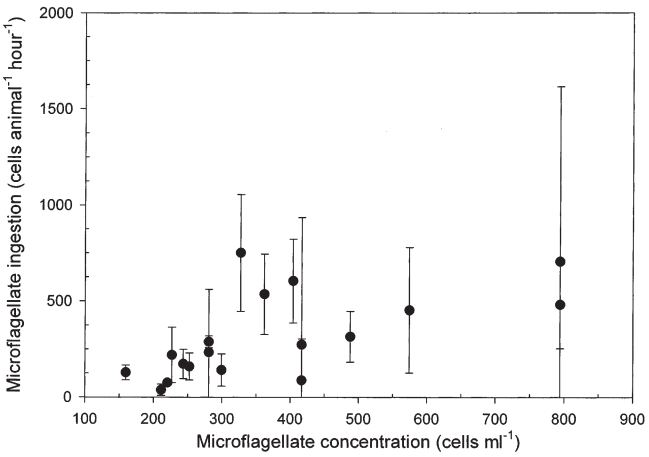

Figure 4 Legend as in Fig. 2 except that data are for microflagellates (<10 μm in size) instead of *Alexandrium* spp.

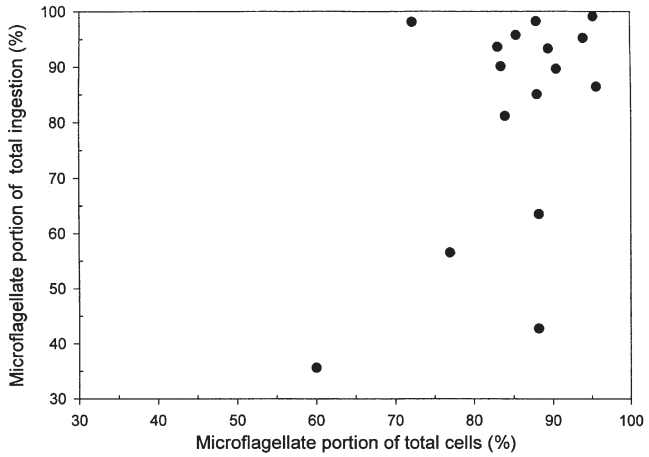

Figure 5 Percentage of total grazing comprised by grazing on microflagellates compared to microflagellate percentage of total phytoplankton abundance.

ducted at levels 0.12–5.11 × 10^3 cells liter^{-1} (Fig. 2), or 0.03–3.93% of total phytoplankton cells present (Fig. 3). Ingestion of *Alexandrium* accounted for only up to 3.2% of total cells ingested (Fig. 3). Phytoplankton assemblages were dominated by athecate microflagellates, and secondarily by diatoms and non-toxic dinoflagellates. Microflagellates were present at abundances of 159.62–793.93 cells mL^{-1} (Fig. 4) and accounted for 60.6–95.56% of total cells (Fig. 5). Grazing on microflagellates accounted for 35.59–98.21% of total grazing (Fig. 5).

Grazing was non-selective, in that slopes of the lines for percentages of total phytoplankton ingested that were comprised by *Alexandrium* spp. and microflagellates, respectively, compared to percentages of total phytoplankton available that were comprised by these taxa (slopes = 0.71 and 1.15, respectively) were not significantly different (r^2 = 0.55, P = 0.0005 and r^2 = 0.26, P = 0.419, respectively) from 1.0 (Figs. 3, 5). There were no apparent adverse effects of *Alexandrium* on grazers during incubations of 18–24 hours, and grazer survival was 100%.

In summary, antipredation effects of high concentrations of *Alexandrium* on some grazers that have been reported from some laboratory studies may only rarely occur during toxic blooms in nature, due to low concentrations of, and consequently low grazing on, this dinoflagellate, and dilution of toxicity by ingestion of other food sources.

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Accumulation of PSP Toxins in the Copepod *Calanus finmarchicus* Feeding on the Toxigenic Dinoflagellate *Alexandrium* Species in Laboratory and Field Studies

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Abstract

Laboratory studies indicated that feeding history of *Calanus finmarchicus* affected clearance rates on *Alexandrium* and that toxin retention efficiencies were low, on the order of 1% to 2% of total toxin ingested. In field studies, *C. finmarchicus* had reduced clearance rates on *Alexandrium* spp. at high concentrations, which indicated avoidance since ingestion rates were low. Still, toxin accumulation was significant in both field-collected copepods that were actively feeding on *Alexandrium* species in surface waters and in non-feeding resting copepods at depth. Our findings suggest that *C. finmarchicus* populations that feed on toxic *Alexandrium* spp. can pose toxicity risks to higher trophic levels.

Introduction

The large-bodied copepod, *Calanus finmarchicus,* is an important food source for higher trophic levels, including fishes and whales, in the North Atlantic. In particular, this species is the dominant copepod in the Gulf of Maine and Bay of Fundy where PSP toxin-producing dinoflagellates of the genus *Alexandrium* cause recurrent blooms. Laboratory and field studies were conducted in order to determine feeding behavior and toxin accumulation in this copepod when feeding on toxic *Alexandrium species.*

Materials and Methods

Laboratory Studies All experiments were conducted with adult female *Calanus finmarchicus* that had been reared in the laboratory at 8°C following the methods described in Campbell *et al.* (2001). Phytoplankton cultures (*Alexandrium fundyense,* clone: GTCA28, toxicity 34–37 pg STX eq. cell^{-1}; *Heterocapsa triquetra,* clone: HT984, non-toxic) were maintained at 16°C on 12:12 L:D cycle and were kept in log-phase growth using semi-continuous culturing techniques (Campbell *et al.* 2001).

1) Effects of feeding history on clearance rate Grazing experiments were conducted with copepods that had either never encountered *Alexandrium* (naïve) or were offered it in mixed cultures (50:50 mixture by biomass of *A. fundyense* and *H. triquetra*) for two weeks prior to the experiment (experienced). Both groups were offered mixtures of toxic *Alexandrium fundyense* and non-toxic *Heterocapsa triquetra* in which the total carbon concentration was held constant (300 µg/L) and the relative proportions of the mixture were varied. For each mixture, duplicate experimental bottles (500-mL) containing 10 copepods each were incubated for 24-hr on a plankton wheel. Control bottles without grazers were used to estimate phytoplankton growth rates. Initial and final samples of phytoplankton were preserved in 1% Lugol's solution for later microscopic counts. Clearance rates were calculated according to the equations of Frost (1972).

2) Short-term feeding and toxin accumulation Copepods were fed a 50:50 mixture, based on carbon (300 µg/L), of *A. fundyense* and *H. triquetra* and their ingestion rates and toxin content monitored every six hours for two days. About 120 copepods were added initially to each of three 8-L experimental containers. Control containers without copepods were also used. Containers were mixed by hand at three-hour intervals to prevent stratification of the phytoplankton. At each time interval, five copepods were sampled from each container for toxin immediately (full) and five copepods allowed to evacuate their gut contents of all toxic material (empty) by feeding on non-toxic *H. triquetra* for one hour prior to sampling. Copepods were frozen for later analysis of toxin composition by high performance liquid chromatography with fluorescence detection (HPLC-FD) as described in Teegarden *et al.* (2003). In addition, grazing estimates were made at each sample interval.

Field Studies were conducted during July 2001 and 2002 in the Grand Manan Basin in the Bay of Fundy.

3) Grazing experiments During 2002, experiments were conducted with late-stage *C. finmarchicus* copepodids that were offered a natural assemblage (ambient) collected from the surface on three occasions and a single concentration-gradient experiment with a natural assemblage spiked with *A. fundyense.* Experimental details were the same as those described for the laboratory grazing experiments.

4) Toxin accumulation During 2001, *C. finmarchicus* (stage C5) were collected for size and toxin analysis with a Multiple Opening-Closing Net Environmental Sensing System (MOCNESS). Samples were collected from an actively growing surface population and a deep resting population. For toxin analysis, 300 copepods from the surface and bottom nets were sorted and divided evenly into triplicate vials and frozen. For carbon and nitrogen analysis, 25 copepods from each net were video taped for length and oil sac volume estimates, placed in tin boats and dried over desiccant. Further details are given in Durbin *et al.* (2002).

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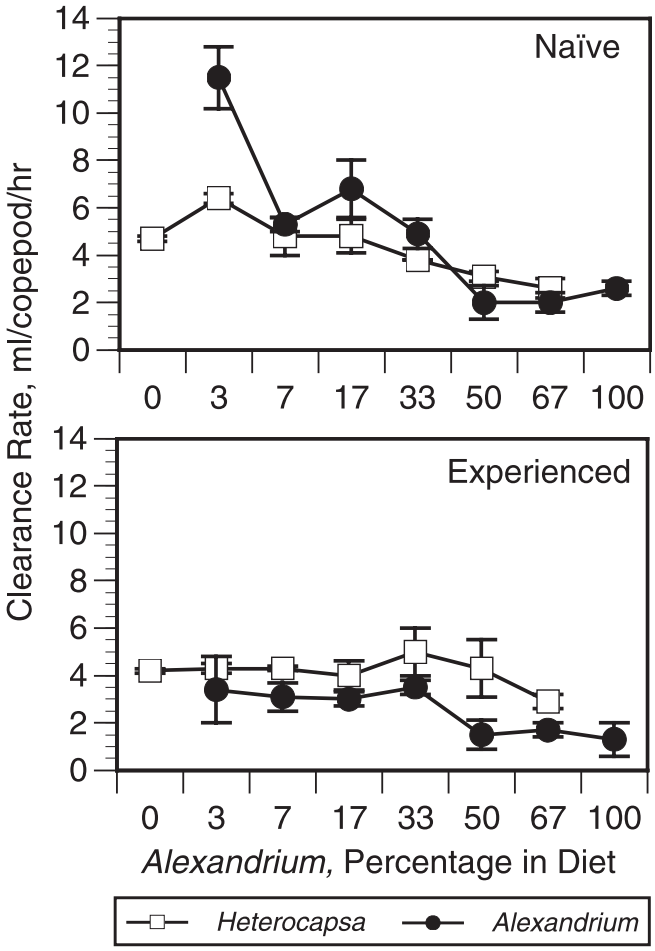

Figure 1 Clearance rates of *C. finmarchicus* adult females on mixed algal cultures in which the percentage of toxic *A. fundyense* in the diet was varied. Range of values indicated and where not shown, range smaller than symbol.

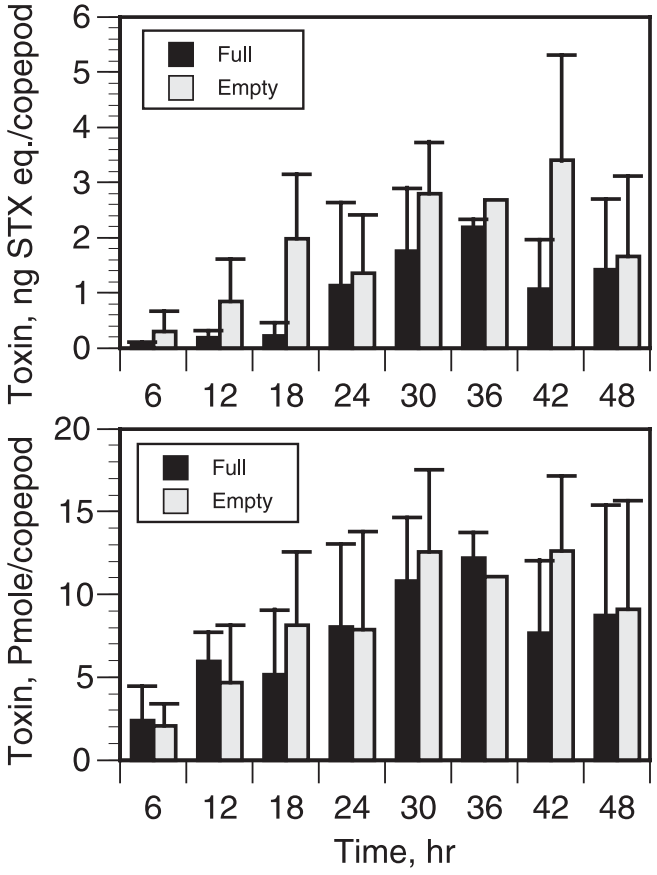

Figure 2 Toxin accumulation in *C. finmarchicus* adult females. Standard errors are indicated.

Results and Discussion

Laboratory Studies

Effects of feeding history on clearance rate Feeding history appeared to influence algal prey preferences (Fig. 1). Naïve copepods preferred the larger *Alexandrium* cells at low concentrations, but experienced copepods always preferred the non-toxic *Heterocapsa.*

Short-term feeding and toxin accumulation. Toxin accumulation in copepod body tissues increased over the first 24–30 hr and then leveled off (Fig. 2). The toxicity (ng STX eq.) of the "empty" copepods was curiously higher than the "full" copepods for the first 18 hr; however, the total toxin concentration (Pmol) was similar. The higher toxicity was apparently caused by higher relative proportions of potent GTX toxins, currently unexplained, in the tissues of 'empty' copepods (Fig. 3). After 24 hr, the toxin profiles were similar. In these experiments, toxin retention efficiencies were very low, on the order of 1–2%.

Field Studies

Grazing experiments The clearance rates of *C. finmarchicus* late-stage copepodids on *Alexandrium* species. decreased dramatically with increased *Alexandrium* concentration (Fig. 4). Similar results were found in experiments with ambient concentrations and the single concentration gradient experiment. This feeding behavior appeared to be due to avoidance and not satiation, since ingestion rates were very low.

Toxin accumulation The smaller, actively growing and feeding surface population contained much higher levels of toxin than the deeper, resting copepods (0.89 vs. 0.41 ng STX eq. copepod^{-1}). Still, toxin levels in resting copepods were significant. The resting population contained much lower levels of the potent GTX toxins than the surface population, which was the reason for their lower toxicity (Fig. 5).

Conclusions

1) Feeding behavior of *Calanus finmarchicus* fed toxic *Alexandrium species* is affected by feeding history. 2) In the Bay of Fundy where *Alexandrium* spp. can be the dominant component of the bloom assemblage, *Calanus* appears to have reduced feeding rates on *Alexandrium* at high concentrations. This is in contrast to our findings in the western Gulf of Maine where *Alexandrium* is a minor component of the bloom assemblage and *Calanus* feeds non-selectively

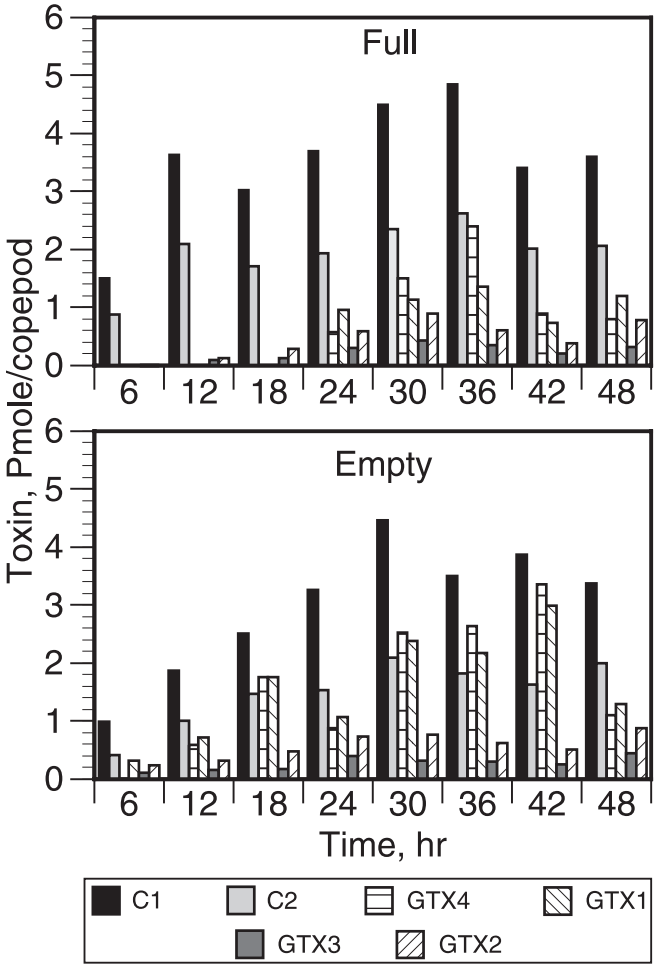

Figure 3 Toxin profiles of *C. finmarchicus* adult females collected at each time interval.

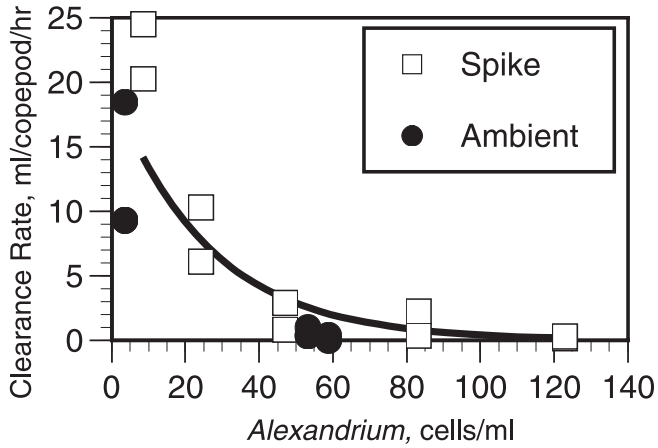

Figure 4 Clearance rates of *C. finmarchicus* late copepodids on *Alexandrium* species from three separate experiments with ambient water and from a single concentration gradient experiment where the ambient water was "spiked" with *A. fundyense.* Exponential curve fit shown for concentration gradient experiment (r^2 = 0.85).

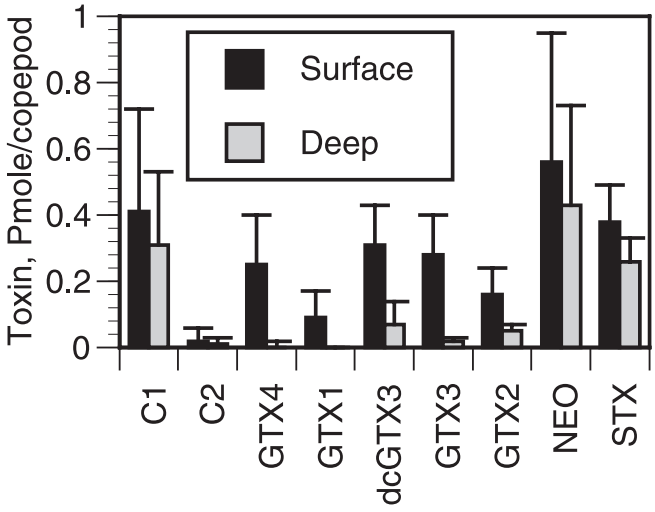

Figure 5 Toxin profiles of *C. finmarchicus* C5 collected from an actively growing surface population and a deep resting population. Standard errors are indicated.

when diatoms are not abundant (see Teegarden *et al.,* 2001). 3) Toxin retention efficiency is low, but toxin accumulation can be significant, posing risks to higher trophic levels including fishes and whales, which feed on *Calanus* (See Durbin *et al.,* 2003).

Acknowledgements

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Differences in the PSP Toxin Profiles of *Mytilus edulis* During Spring and Autumn Blooms of *Alexandrium tamarense* Off Mar Del Plata Coast, Argentina

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Abstract

A bimodal Paralytic Shellfish Poisoning (PSP) cycle was observed during a study conducted at a permanent station off Mar del Plata during 2000. Unlike previous records, in which the autumn toxicity was caused by advection of *Gymnodinium catenatum* cells from the coastal area of the Río de la Plata, during the spring and autumn of 2000, toxicity was caused by *Alexandrium tamarense.* Although the maximum toxin content in shellfish was similar during the autumn and spring, the toxin profiles were remarkably different. This contrast in toxin composition, together with some morphological differences in *A. tamarense* cells between the spring and autumn, suggest the alternate predominance of two genetically different *A. tamarense* populations.

Introduction

Previous studies of Paralytic Shellfish Poisoning (PSP) toxicity in the mussel *Mytilus edulis* from the coastal waters off Mar del Plata have, in some years, shown a main peak in spring and a secondary peak in autumn. While PSP toxicity in spring is due to *Alexandrium tamarense* (Carreto *et al.,* 1998), the origin of the autumnal toxicity has been related to the advection of *Gymnodinium catenatum* cells from the coastal area of Río de la Plata (Akselman *et al.,* 1998). This paper reports on the seasonal appearance of *A. tamarense* and *G. catenatum* during a study of the annual (2000) phytoplankton cycle and the associated *Mytilus edulis* toxicity at a permanent station off Mar del Plata. Results showed that the peak mussel toxicity detected in early spring and early autumn is related to the growth of two different *A. tamarense* populations.

Materials and Methods

Sampling was done during one-day cruises to the permanent monitoring station off Mar del Plata (EPEA, 38°28′S 57°41′W) at biweekly (during spring and autumn) to monthly intervals, between February 2000–February 2001. Continuous vertical recordings of temperature and salinity were obtained with a CTD (Sea-Bird Electronics). Light penetration (PAR, UVA, and UVB) and natural fluorescence profiles were obtained with a PUV-500/510B (Biospherical Instruments). Water samples were taken from the surface with a bucket and from different depths using Niskin bottles. Vertical hauls were used to obtain qualitative phytoplankton samples. Mussel samples (*Mytilus edulis*) were taken from the benthic community at the EPEA station (45 m depth) using a dredge. Chlorophyll-*a* concentration was determined using the fluorometric method. Nutrient concentrations were determined by standard techniques using a Technicon TA II Autoanalyzer. Phytoplankton composition was identified and quantified using the Utermöhl (1958) method. PSP mussel toxicity was tested by the AOAC mouse bioassay method, and PSP toxin composition was analysed by HPLC using the three-step isocratic elution method [Oshima, 1995]. Dr Y. Oshima kindly provided standard solutions.

Results and Discussion

Surface temperature followed the seasonal cycle characteristic of this area, varying from 10.0°C in August-September to 21°C in late summer, when the highest development of the seasonal thermocline was recorded (Fig. 1a). Salinity variations in the water column were also marked (33.6–34.1 psu). During most of the year, highly saline waters prevailed (>33.9 psu). Low surface values (33.6 psu), indicative of the influence of Río de la Plata coastal waters, were recorded during the summer (Fig.1b). Nitrate concentrations varied from 0.5 μM at the surface in the summer to 5.0 μM in the winter following the seasonal formation and breakdown of the pycnocline (Fig. 1c).

The chlorophyll-*a* distribution at the EPEA station showed a seasonal cycle, which approximately followed the pattern of stratification and mixing of the water column (Fig. 2a). Chlorophyll-*a* concentrations were low in the summer. The breakdown of the thermocline gave rise to an early fall bloom dominated by the diatoms *Nitzschia, Hemiaulus* and *Thalassiosira* and the silicoflagellate *Dictyocha octonaria.* At the beginning of the bloom, two toxic dinoflagellates, *A. tamarense* (Fig. 2b) and *G. catenatum* (Fig.

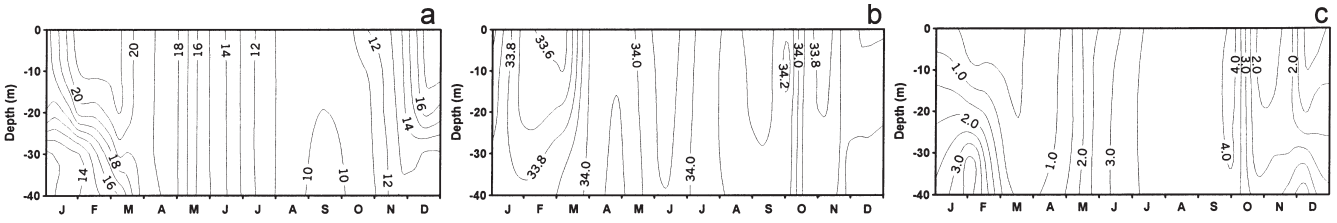

Figure 1 Time-space variation of **a** Temperature (°C), **b** Salinity (psu), and **c** Nitrate (mM) at the EPEA.

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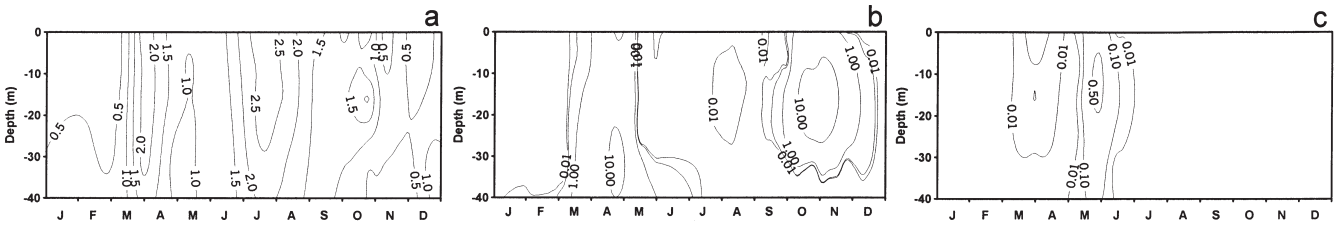

Figure 2 Time-space variation of: **a** Chlorophyll-*a* (mg L^{-1}), **b** *A. tamarense* abundance (cells 10^3 L^{-1}), and **c** *G. catenatum* abundance (cells 10^3 L^{-1}) at the EPEA.

2c) were present in relatively low amounts near the surface. By mid-April, an *A. tamarense* bloom had developed, reaching values of up to 17,000 cells/L in the top two meters. Concentrations of *A. tamarense* cells were high through the water column, with a second peak near the bottom (14,000 cells/L) (Fig. 2b). In contrast, *G. catenatum* was observed only in patches of low cell concentrations (<1000 cells/L) (Fig. 2c). Vegetative cells of *A. tamarense* and *G. catenatum* disappeared from the EPEA station by mid-May, when diatoms were again the main component of the phytoplankton community. In mid winter an unusually high chlorophyll-*a* concentration (Fig. 2a) was ascribed to the diatoms *Meunieria, Leptocylindrus* and *Ditylum.* Towards the end of winter and the beginning of spring, as corroborated by previous studies in this area (Carreto *et al.,* 1998), *A. tamarense* cells were again found near the surface in a community dominated by the diatoms *Leptocylindrus, Pseudo-nitzschia,* and *Eucampia.* By mid-October, concentrations of *A. tamarense* increased throughout the water column, reaching values of up to 37,000 cells/L in the 10-meter sample (Fig. 2b). The bloom condition persisted until early November when *Noctiluca scintillans* cells were observed grazing on *A. tamarense.* Vegetative cells of *A. tamarense* practically disappeared from the EPEA station by mid-November. During the spring bloom, *A. tamarense* occurred principally as solitary cells. Autumn populations had the distinctive and unusual ability to form long, fast-moving chains of up to eight cells. Thecal analysis of these chain-forming cells showed the morphological characteristics of *A. tamarense.* Diagnostic characters in plates such as the pore plate (Po) and the sulcal posterior plate (Sp) ruled out other chain-forming species such as *A. catenella* and *A. fraterculus;* the latter species is distributed in the shelf area under study (Balech, 1995).

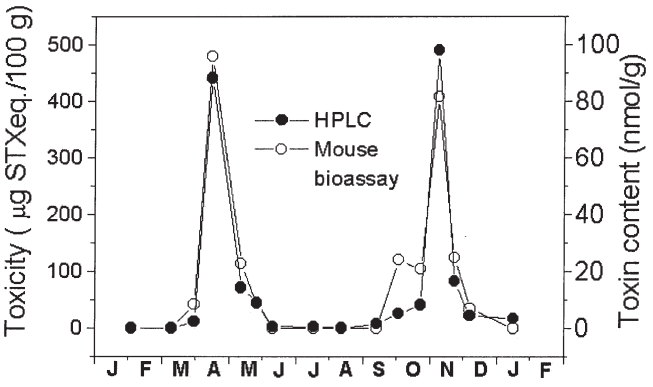

Figure 3 Temporal evolution of *M. edulis* toxicity.

In agreement with the annual variation of *A. tamarense* abundance, the temporal evolution in mussel toxicity showed a bimodal cycle with maximum values during autumn and spring (Fig. 3). The values of PSP found in autumn were low in comparison to the typical spring values (Carreto *et al.,* 1998). However, the 2000 cycle was anomalous, in that the autumn toxicity was slightly higher than that observed during spring. In both events, mussels became toxic during bloom initiation and showed the highest toxicity one month later, coinciding with the maximum development of the bloom. During the immediate post-bloom period when *A. tamarense* cell numbers declined to <100 cells/L, the total toxin content in the mussels decreased dramatically. During the initial phase of rapid detoxification, the half-life for

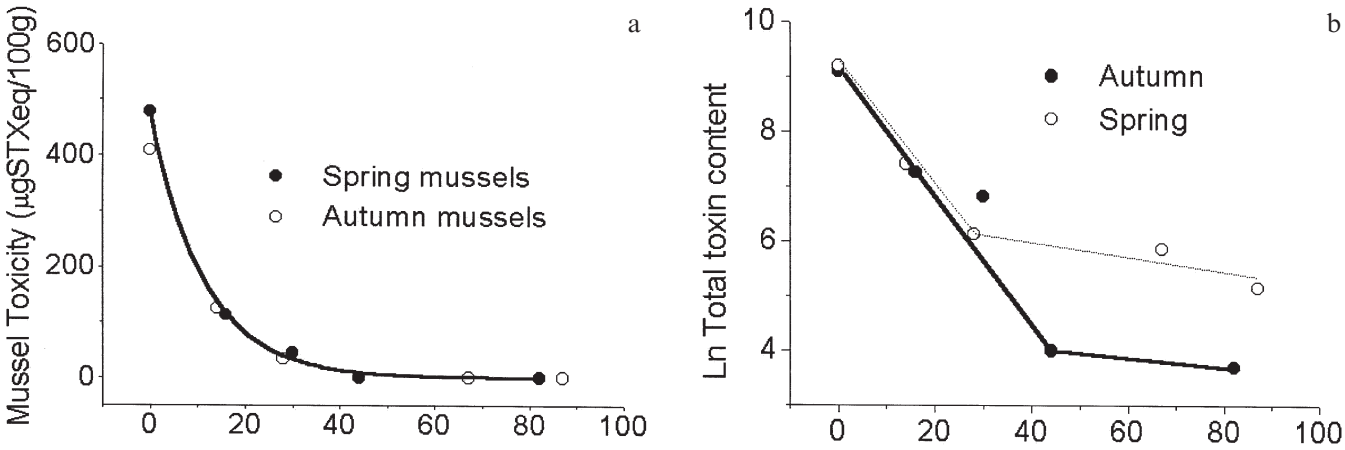

Figure 4 Detoxification trajectories in *M. edulis* fitted by **a** monophasic and **b** biphasic exponential curves.

PSP toxicity was similar in both events (about eight days). Mussel toxicity dropped to regulatory limits within two weeks following the disappearance of *A. tamarense* (Fig. 4a). This initial rapid toxin loss was followed by a period of slower detoxification rate of the remaining toxins (Fig. 4b).

In both blooms, the toxin profile of *M. edulis* was dominated by the carbamate toxins GTX4, GTX1, GTX2 and GTX3, and to a lesser extent the N-sulfocarbamoyltoxins C2/C1, which together constituted >80% of the total toxin composition. As minor components, STX and neo-STX were also found (Fig. 5). While the agreement between the toxin profiles of the mussels compared to those of the *A. tamarense* regional isolates (Carreto *et al.,* 1996, 2001; Mendez *et al.,* 2001) was not perfect, there is sufficient similarity to establish significant relationships. The discrepancy in the toxin composition can be explained by compositional shifts resulting from differential toxin retention and toxin bioconversions within the shellfish (Oshima, 1995).

During the toxin uptake phase of the blooms, while total toxin concentrations continued to increase, there was a significant decrease in the relative concentration of the C2/C1 epimeric pair, especially in the autumn mussels (Fig.6a). There was also a significant decrease in the epimeric ratios GTX4 : GTX1 and GTX3 : GTX2. During the high detoxification rate period, the ratios GTX4 : GTX1 and GTX3 : GTX2 decreased substantially in both blooms, but the relative amount of STX and neo-STX remained relatively constant. The most dramatic change was observed during the period of the slower detoxification rate, as the remaining low PSP toxin levels were enriched with STX and the GTX2/GTX3 epimeric pair (Figs. 6a,b).

Although changes in the toxin composition of the mussels took place during the uptake and detoxification periods, the percent composition of each toxin was significantly different between the spring and autumn populations for all sequences except for the period of low detoxification rate. The ratio of GTX4, 1/GTX2, 3 accounted for most of the discrimination between the two populations.

In conclusion, the initiation of *A. tamarense* growth started in early spring and early autumn under very different environmental conditions, and simultaneously with the beginning of diatom blooms. The ability to form blooms under different environmental conditions and the distinctive toxin profiles and morphology of spring and autumn populations suggest some genetic heterogeneity between *A. tamarense* populations.

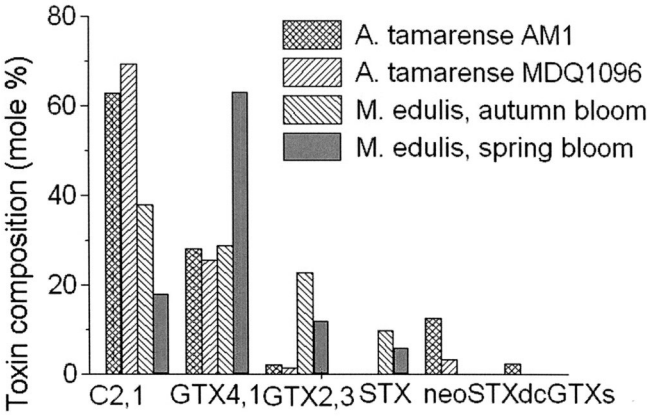

Figure 5 Mole % toxin composition of *A. tamarense* and shellfish extracts.

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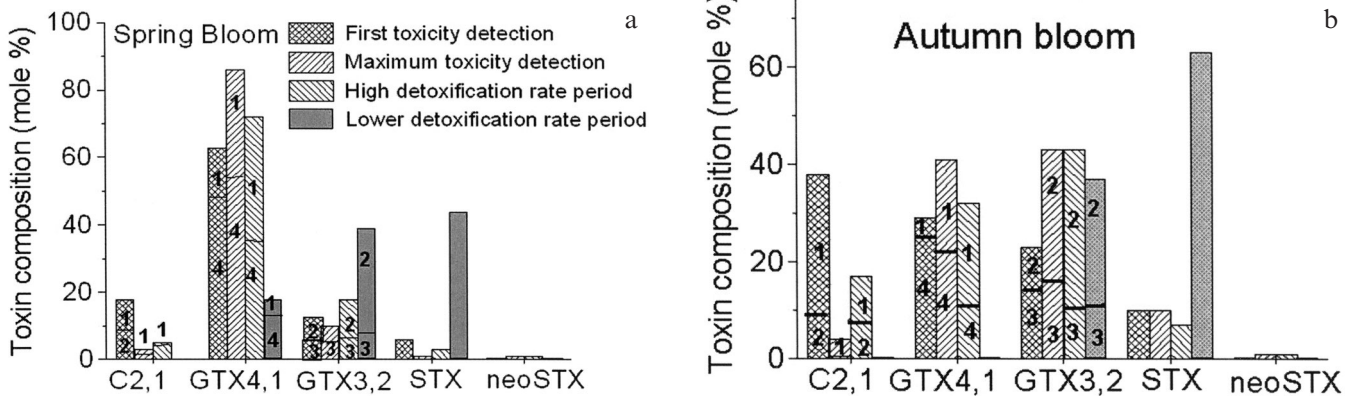

Figure 6 Changes in the toxin composition of mussels during the detoxification period. **a** Spring bloom and **b** Autumn bloom.

Reproductive Success of the Copepod *Acartia clausi* During a Bloom of *Dinophysis* spp.

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Abstract

The effect of *Dinophysis* spp. blooms on the reproductive success of the copepod *Acartia clausi* was studied. Egg and naupliar production was positively correlated with food availability, whereas hatching success was negatively correlated with *Dinophysis* spp. abundance. *Acartia clausi* did not feed on *Dinophysis* spp., suggesting that the reduced hatching success observed at high densities of *Dinophysis* spp. was not due to a negative effect of toxic cells. The oceanographic conditions that favour an increase in *Dinophysis* spp. abundance were not favourable to the abundance of those phytoplankton species used as food by copepods, indicating that the reduced hatching success was probably due to a reduction in food availability.

Introduction

Copepods feeding on toxic dinoflagellates usually exhibit reduced food intake, food assimilation and/or fecundity. However, it is uncertain which of these deleterious mechanisms could affect copepod fitness in the field. The biochemical composition of the food mixture affects both egg production and hatching success in copepods (Koski *et al.,* 1998; Guisande *et al.,* 2000). Laboratory studies have shown that marine toxic dinoflagellates might cause changes in the species composition of the phytoplankton community (Blanco and Campos, 1988; Arzul *et al.,* 1999). Therefore, if the composition of copepod food assemblages is affected by toxic dinoflagellates, then copepod reproductive success could be negatively affected due to nutritional inadequacy. The copepod *Acartia clausi* and the toxic dinoflagellates *Dinophysis* spp. usually co-occur in the Ría de Pontevedra (NW Spain). The aim of this study was to determine whether a bloom of *Dinophysis* spp. interfered with the reproductive success of *A. clausi.*

Materials and Methods

Phytoplankton and Zooplankton Collection Samples were collected weekly from 3 May to 17 August 1999 at stations P2 (42°21.40′N, 8°46.42′W), P3 (42°24.02′N, 8°43.93′W) and P8 (42°20.40′N, 8°47.50′W) (map shown in Maneiro *et al.,* 2000). Sampling was carried out by means of a hose (Lindahl, 1986) divided into three depth strata (0–5, 5–10 and 10–15 m), and by vertically integrated tows from 20 m depth.

Hydrographic Sampling Hydrographic sampling was carried out by CTD cast. The Brunt-Väisälä or buoyancy frequency equation was used to measure the strength of the density gradients:

$$N = \sqrt{\frac{g}{\bar{\rho}}\frac{d\rho}{dz}} \qquad S = \frac{\int_{z_1}^{z_n} Ndz}{\int_{z_1}^{z_n} dz}$$

where ρ is the depth water density (in kg m^{-3}), $\bar{\rho}$ is the mean ρ of the water column, g is the acceleration due to gravity and z is the depth. The integrated buoyancy frequency estimated by the trapezoid method was used as an indicator of stability of the water column (*S*).

Estimation Food Availability Seawater collected at 2, 5, 10, 15 and 20-m depths at the field stations was mixed and sieved to analyse total protein concentration of the seston in 3–20 µm and 20–100 µm fractions. Analyses were performed on GF/C-filtered material using the method described by Lowry *et al.* (1951) and modified by Markwell *et al.* (1978). From protein analysis and determination of the phytoplankton species abundance at the stations, a stepwise regression was carried out to determine the main phytoplankton genera that contribute to the protein content. The sum of the abundance of these genera was called food abundance.

Egg and Naupliar Production and Hatching Success 20–40 females from each station were transferred individually to 25-mL beakers containing the seston size fraction 3–20 µm. Copepods were kept at 15°C. After 24 h, eggs were collected and counted. Three replicates of 60–150 eggs were incubated for a further 48 h before fixation, and the hatched nauplii were counted.

Grazing Experiment Food was collected at P3 on 18 May by vertical net hauls from 20-m depth with a 20-µm-mesh net. After having passed through a 75-µm mesh net, the seston was concentrated on a 20-µm mesh size net. It was settled for 2 h, and the upper part of the water was then siphoned off to collect swimming dinoflagellates for the grazing experiment. Four different experimental food concentrations were used at the range of *Dinophysis* spp. abundance observed at the station during the period (between 643 and 4,325 cells L^{-1}). For 24 h, seven replicates of 25-mL experimental jars with one adult female and seven controls were kept at 15°C for each experimental food concentration. The model of Frost (1972) was used to estimate ingestion rates.

Results and Discussion

Figure 1 shows distribution of σ_t (ρ – 1,000) and *S* during the sampling period. The lowest σ_t values and the highest

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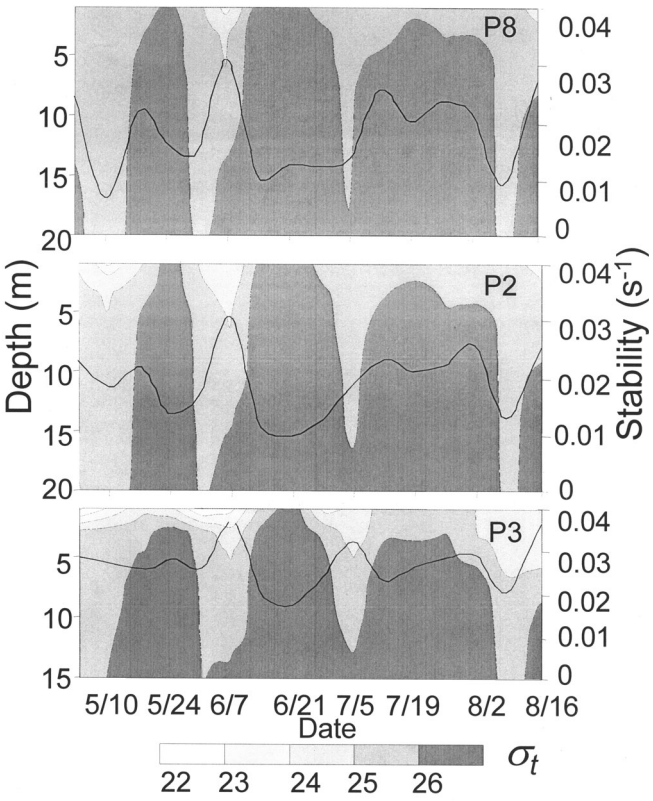

Figure 1 Distribution of σ_t and stability (in s^{-1}, solid line) at the stations during the sampling period.

S in the water column were found at P3, which is the inner sampling station. At the nearest station to the mouth (P8), the highest σ_t values and the lowest *S* were observed. *Dinophysis* spp. abundance at the sampling stations is shown in Fig. 2.

In agreement with Maneiro *et al.* (2000), the grazing experiment showed that *A. clausi* did not feed on *Dinophysis*

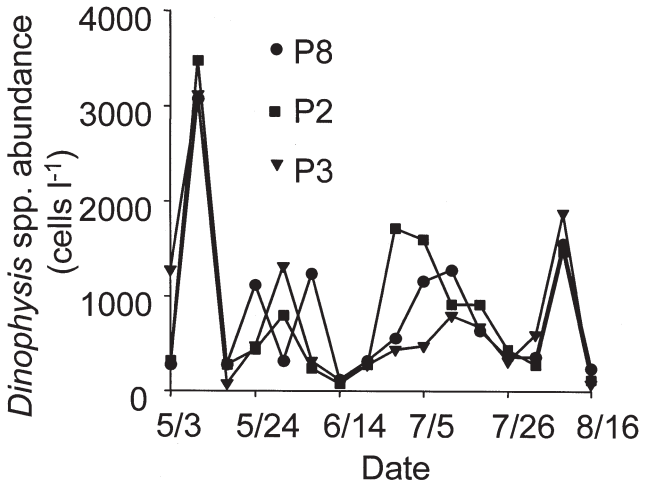

Figure 2 *Dinophysis* spp. abundance during sampling period.

spp. (ANOVA, $F_{3,24} = 0.191$, $P = 0.901$). The mean value of ingestion was 1.2 ± 2.1 cells copepod^{-1} d^{-1} (mean ± SE, n = 28).

Protein content of the size fraction 3–20 µm in the water column was the only variable related with both egg production and naupliar production (Fig. 3). However, hatching success was also significantly related with total protein of the seston size fraction 3–20 µm and *Dinophysis* spp. abundance (Fig. 4) ($F_{2,15} = 41.3$, $r^2 = 0.85$, $P < 0.001$).

Because egg hatching success in copepods is sensitive to the chemical composition of the maternal diet (Laabir *et al.,* 1999; Guisande *et al.,* 2000), the negative effect observed could be due to changes in the phytoplankton assemblage. In fact, over a threshold concentration of 500 cells l^{-1}, a reduced food concentration was observed as the dinoflagellate abundance increased (Fig. 5, $F_{1,20} = 8.5$, r^2=0.3, $P = 0.009$). DSP toxins can act as an allelopathic compound that affects phytoplankton growth (Windust *et al.,* 1996). However,

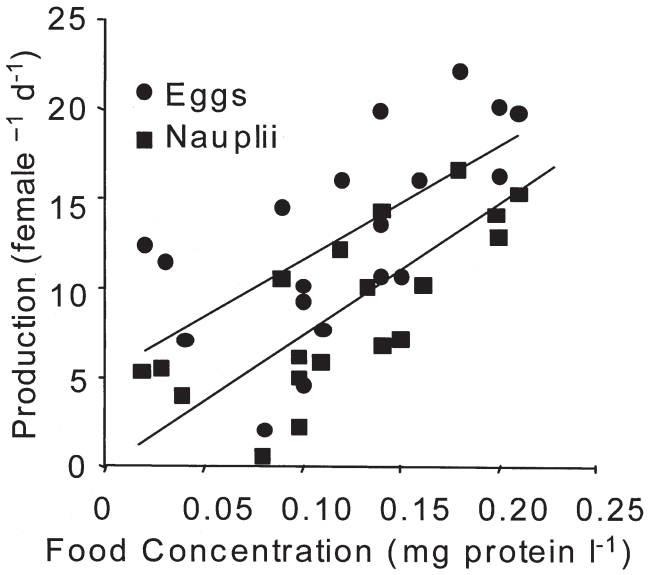

Figure 3 Relationship between food concentration and egg and naupliar production.

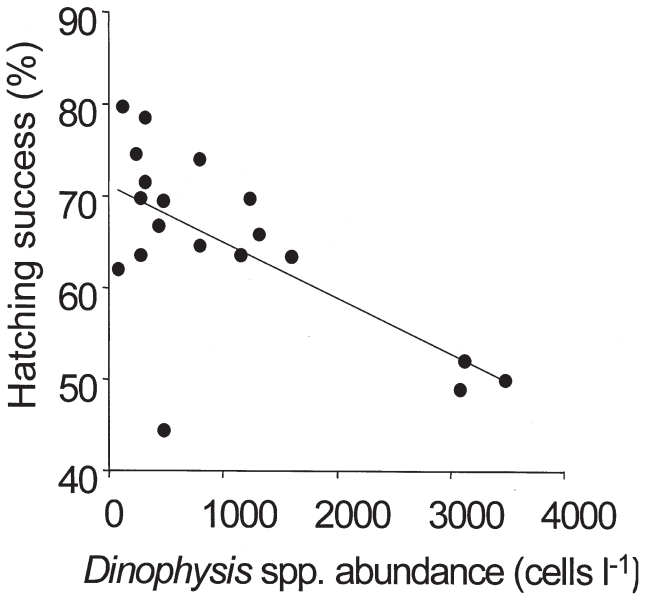

Figure 4 Relationship between egg hatching success and *Dinophysis* spp. abundance. All standard errors were lower than 14.6.

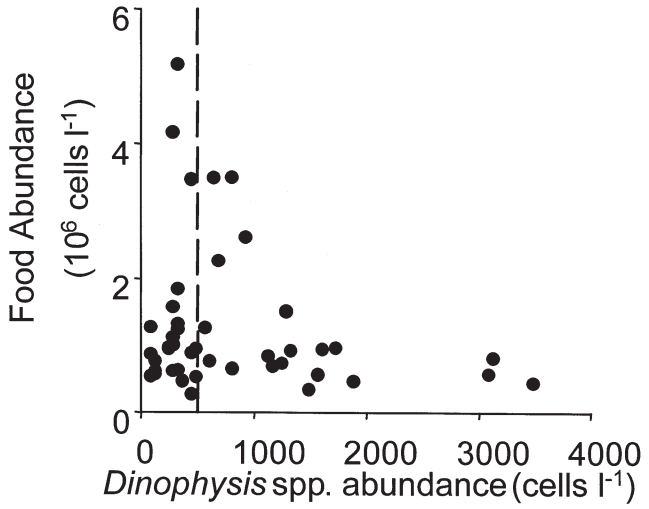

Figure 5 Relationship between food abundance and *Dinophysis* spp. abundance. Vertical dotted line shows the *Dinophysis* spp. abundance of 500 cells L^{-1}.

the fact that copepods did not feed on this toxic genus indicates that DSP toxins do not have an important influence on egg development.

During this study, changes in the abundance of both *Dinophysis* spp. and food seemed to be explained by the prevailing oceanographic conditions. Over the threshold concentration of 500 cells l^{-1}, a stepwise regression indicated that σ_t and S were the main factors that determined the abundance of both *Dinophysis* spp. ($F_{2,18} = 14.5$, $r^2 = 0.62$, $P < 0.001$) and food ($F_{2,18} = 19.5$, $r^2 = 0.68$, $P < 0.001$). *Dinophysis* spp. was higher at low values of S and σ_t of the water column (Fig. 6a). These results are in agreement with those studies that have shown that *Dinophysis* spp. toxic events in Galician Rias occur during warm periods (Reguera *et al.,* 1993, 1995; Blanco *et al.,* 1998). Contrary to *Dinophysis* spp., food abundance was higher at medium and high values of stability and stratification of the water column (Fig. 6b). Therefore, when the abundance of *Dinophysis* spp. was high, as copepods avoid feeding on toxic dinoflagellates and the food abundance was low, copepod starvation seems to be the reason for the reduced egg hatching success observed in *A. clausi* during this bloom of *Dinophysis* spp.

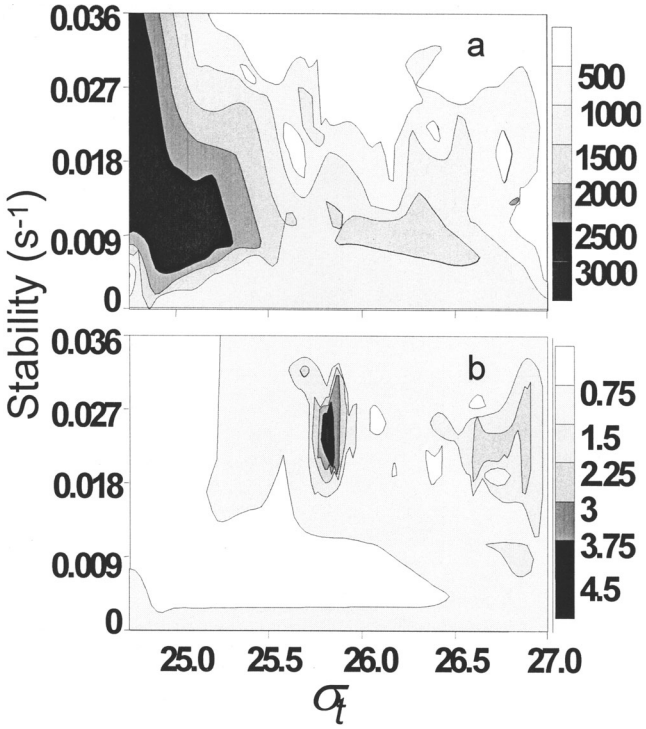

Figure 6 Distribution of **a**) *Dinophysis* spp. abundance (in cells L^{-1}) and **b**) food abundance (in 10^6 cell L^{-1}) at the stations as a function of σ_t and stability of water column.

Acknowledgements

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Survival and Reproduction of the Copepod *Parvocalanus crassirostris* Fed the Texas Brown Tide Alga *Aureoumbra lagunensis*

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Abstract

Previous studies have shown that several species of zooplankton will not feed on the Texas brown tide alga *Aureoumbra lagunensis,* due perhaps to its small size, low nutritional value and/or toxicity. The calanoid copepod *Parvocalanus crassirostris* feeds effectively on phytoplankton in the size range of the brown tide. Studies were conducted on survival, egg production and egg hatching success of *P. crassirostris* fed *A. lagunensis,* another similarly sized alga (*Isochrysis galbana*) or nothing at all. Survival of adult copepods was not significantly different on diets of either *Aureoumbra* or *Isochrysis* after one week, whereas copepods held without food starved in a few days. *Parvocalanus crassirostris* produced significantly more eggs on a diet of *Isochrysis* than on an *Aureoumbra* diet, and copepods fed *Aureoumbra* produced significantly more eggs than those held without food. Hatching success of the eggs was also significantly higher for copepods fed *Isochrysis* compared to those fed *Aureoumbra*. When *Parvocalanus* were fed mixtures of *Isochrysis* and *Aureoumbra* in various proportions, egg production and hatching success were higher than expected. This suggests that *Aureoumbra* is nutritionally inferior to *Isochrysis* but not toxic to *Parvocalanus*.

Introduction

The uninterrupted bloom of the Texas brown tide alga *Aureoumbra lagunensis* in the Laguna Madre of south Texas from January 1990 through October 1997 is the longest continuous harmful algal bloom event that has been scientifically documented (Buskey *et al.,* 2001). Extended drought leading to extreme hypersaline conditions and an unusually severe freeze in December of 1989 appear to have disrupted grazer populations contributing to the initiation of the bloom (Buskey *et al.,* 1997). A nutrient pulse released by fish killed in the freeze may have intensified the initial growth of the bloom (DeYoe and Suttle, 1994). The reasons for the extraordinary persistence of this bloom are less clear. Limited water exchange between the Laguna Madre and the Gulf of Mexico reduce losses of cells through advection and field studies have documented that high densities of *A. lagunensis* have adverse effects on grazer populations (Buskey *et al.,* 1998). Laboratory studies have also demonstrated that *A. lagunensis* does not support the growth and reproduction of many zooplankton species as well as other foods of similar size (Buskey and Hyatt, 1995; Liu and Buskey, 2000). For the dominant copepod in Laguna Madre, *Acartia tonsa,* a diet of *A. lagunensis* results in lower survival and reproduction (Buskey and Hyatt, 1995); these effects may result in part from reduced ingestion due to this small alga being outside *A. tonsa*'s preferred size range for food (Berggreen *et al.,* 1988). While no similar detailed studies have been published on the food size preferences of *Parvocalanus crassirostris,* this small calanoid species appears better adapted for feeding on small phytoplankton species. The purpose of this study was to determine if *A. lagunensis* had similar detrimental effects on a copepod species that fed more readily on phytoplankton in this size range, and to help determine if the detrimental effects of a diet of *A. lagunensis* were due to toxic properties of the cells or simply due to its nutritional inadequacy as a food.

Materials and Methods

Cultures of *A. lagunensis* were grown in modified f/2 media (Buskey *et al.,* 1998) and *Isochrysis galbana* were grown in standard f/2 (Guillard and Ryther, 1962). Copepods were collected in the Aransas Ship Channel using a 30 cm diameter, 153 µm mesh plankton net, and adult female *P. crassirostris* were sorted from the plankton sample using a wide-bore pipette. For the survival experiment two *P. crassirostris* females were placed in 70 mL tissue culture flasks with either 0.5 mg C L^{-1} *I. galbana* (control), 0.5 mg C L^{-1} *A. lagunensis* or no food (starved), with ten replicates for each treatment. Flasks were rotated on a bottle roller at 25°C on a 15:9 light-dark cycle. These flasks were observed daily, and the number of live copepods counted. For the egg production experiments, three 1-liter bottles were prepared with each of the following food additions: 0.5 mg C L^{-1} of *I. galbana,* 0.5 mg C L^{-1} *A. lagunensis* or no food. Ten adult female and one adult male *P. crassirostris* were placed in each bottle. These bottles were held under the same conditions as in the survival experiment. After 24 hours the copepods were collected using a 153 µm mesh sieve, counted again and placed in bottles with fresh seawater and food. Twenty-four hours later, the contents of the bottles were passed through a 153 µm mesh sieve to collect copepods and a 20 µm mesh sieve to collect the eggs and nauplii. This experiment was repeated six times for each food treatment. For the egg hatching experiments, the same conditions were used as described in the egg production experiments (except adults fed 1 mg C L^{-1} *I. galbana, A. lagunensis* or a 50:50 mix), but the eggs were placed in cell wells (12 eggs per well in each of six wells) and observed at regular intervals to determine the proportion that hatched. Egg production experiments were repeated following the suggestion of Jónasdóttir *et al.* (1998) using mixtures of poor and nutritious foods to help determine if the poor food is toxic or simply nutritionally inadequate. Mixtures of *I. galbana* (*I.g.*) and *A. lagunensis* (*A.l.*) were used in the following proportions: 100% *I. g.*; 75%

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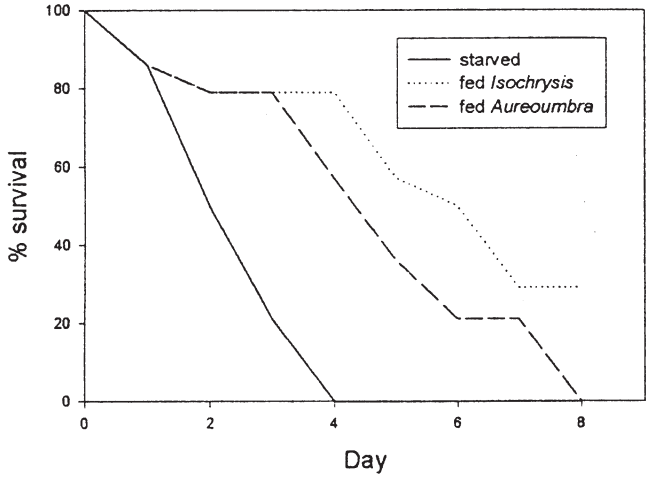

Figure 1 Survival of adult female *Parvocalanus crassirostris* fed a diet of *Isochrysis galbana, Aureoumbra lagunensis* or held without food (starved).

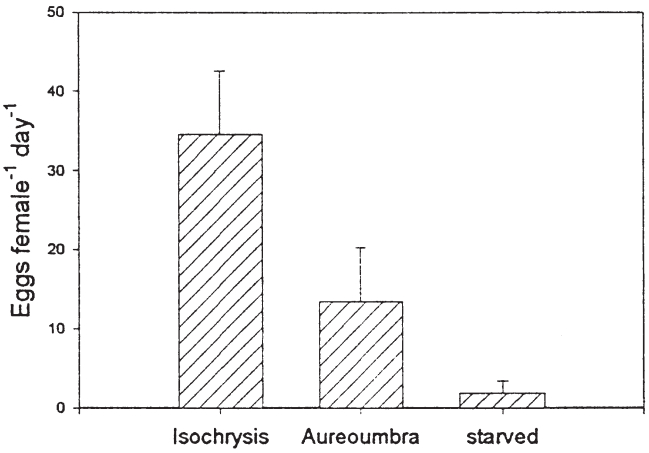

Figure 2 Egg production of adult female *Parvocalanus crassirostris* fed a diet of *Isochrysis galbana, Aureoumbra lagunensis* or held without food (starved).

I. g.: 25% *A. l.*; 50%:50% mix; 25% *I. g.*: 75% *A. l.* and 100% *A. l.* with all mixtures equaling 0.5 mg C L^{-1} total food.

Results

All adult female *P. crassirostris* held without food were dead on day 4 of the survival experiment (Fig. 1). For those fed a diet of 0.5 mgC L^{-1} of *I. galbana* or *A. lagunensis,* 79% of the copepods were alive on day 3, compared to only 21% of the starved group. Survival was ~10–30% lower for copepods fed *A. lagunensis* than those fed *I. galbana* over days 4–7 of the experiment. On day 8, none of the copepods fed *A. lagunensis* survived while only 21% of copepods fed the control diet, *I. galbana,* were still alive. For the egg production experiments with single foods (Fig. 2), there was a significant difference in egg production for each of the food treatments (Fig. 2; Kruskal-Wallis One Way Analysis of Variance on Ranks, $P = < 0.001$). Pairwise multiple comparison procedures (Dunnett's Method) revealed that adult females produced significantly fewer eggs when either fed *A. lagunensis* or starved, compared to the control treatment of copepods fed *I. galbana.* In the egg hatching experiment, *Parvocalanus* fed 100% *A. lagunensis* had 31.8% hatching success compared to 98.3% success when fed 100% *I. galbana* or 85.8% success in a 50:50 mix of the two foods (Fig. 3). When *P. crassirostris* were fed *I. galbana* and *A. lagunensis* either alone or in mixtures, only 4 offspring per female were produced on a diet of *A. lagunensis,* while 39 offspring per female were produced on a diet of *I. galbana.* Offspring production rates increased rapidly as increasing proportions of *I. galbana* were mixed in with the *A. lagunensis* (Fig. 4).

Discussion

The Texas brown tide alga *A. lagunensis* has been previously demonstrated to be a poor food for a variety of zooplankton species (Buskey and Hyatt, 1995). For the copepod *A. tonsa,* the small size of *A. lagunensis* cells (~4 μm diameter) was thought to contribute to low ingestion rates and reduced reproduction and survival (Buskey and Stockwell, 1993). While the Texas brown tide alga *A. lagunensis* is readily consumed by the copepod *P. crassirostris,* it appears to be of poorer nutritional value than the similarly sized alga *I. galbana*. Copepods fed a diet of *A. lagunensis* alone survive twice as long as starved copepods, but have poorer survival than copepods fed a similar amount of *I. galbana* (Fig. 1). In the copepod *Acartia tonsa,* many of those *A. lagunensis* cells that are ingested pass through its digestive track alive, protected by the thick external polysaccharide (EPS) layer (Bersano *et al.,* 2002). Reduced digestion of *A. lagunensis* may contribute to its poor nutritional value to *P. crassirostris*.

Reductions in egg production by copepods fed *A. lagunensis* (Fig. 2) also support the hypothesis that this alga is a poor food for *P. crassirostris*. Numerous studies have demonstrated the influence of food quality and quantity on copepods egg production rates (*e.g.,* Checkley, 1980; Ambler, 1986). Individuals fed *A. lagunensis* produced significantly more eggs than copepods held without food, but fewer eggs than copepods fed *I. galbana*. The small size of *A. lagunensis* alone does not appear to be a limiting factor for fecundity of *P. crassirostris;* copepods fed the similarly sized *I. galbana* produced more than 30 eggs per female per day. In contrast, *Acartia tonsa* does poorly on a diet of small cells. The egg production of *A. tonsa* fed *A. lagunensis* was not significantly different from that of starved individuals, and was less than one third of maximum fecundity when fed the similarly sized *I. galbana*. Several recent studies have demonstrated that changes in egg production rate alone may not provide complete insight into the effects of potentially harmful algae on zooplankton population dynamics; the hatching success of the copepod eggs must also be considered (Poulet *et al.,* 1994; Miralto *et al.,* 1999). In this study we found that a maternal diet of *A. lagunensis* also reduced the hatching success of *P. crassirostris* eggs

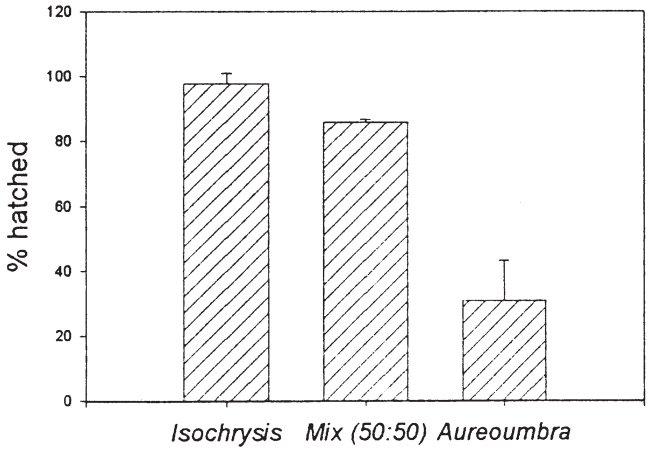

Figure 3 Egg hatching success for newly laid eggs from female *Parvocalanus crassirostris* fed a diet of *Isochrysis galbana* or *Aureoumbra lagunensis.*

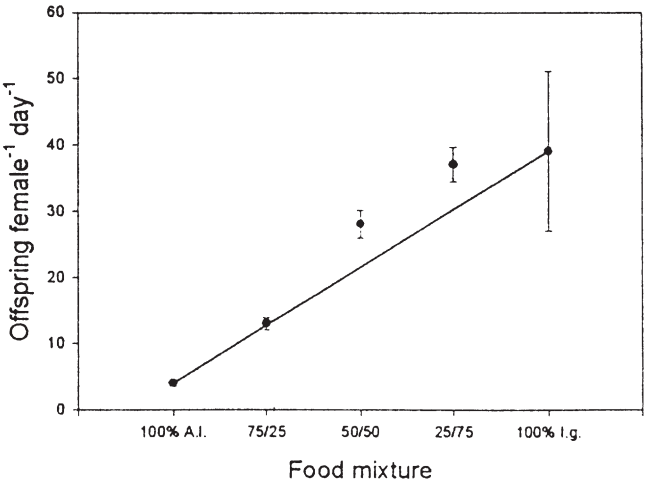

Figure 4 Egg production of adult female *Parvocalanus crassirostris* fed mixtures of *Isochrysis galbana* and *Aureoumbra lagunensis* in various proportions.

(Fig. 3). This raises the issue of whether a diet of *A. lagunensis* is merely nutritionally inadequate for *P. crassirostris* or may be directly toxic to it. The protocol of Jónasdóttir *et al.* (1998) is designed to differentiate between potentially toxic diets and those that are simply nutritionally inadequate. Based on this procedure, since egg production and hatching success increased rapidly as the proportion of *I. galbana* in the diet increased (Fig. 4), it suggests that *A. lagunensis* is only nutritionally inadequate, rather than toxic to *P. crassirostris*. Although a diet of *A. lagunensis* does not impact the survival and reproduction of *P. crassirostris* as severely as they do *A. tonsa,* the reduced fecundity would have a negative impact on populations during a brown tide bloom. Reductions in grazer populations may play an important role in the extraordinary persistence of brown tide blooms.

Acknowledgements

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