HARMFUL ALGAE 2012

15차 국제 적조회의보고서

The 15th International Conference on Harmful Algae

October 29 - November 2, 2012, CECO,
Changwon, Gyeongnam, Korea

EDITORS

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Joong Ki Choi

INTERNATIONAL SOCIETY FOR THE STUDY OF HARMFUL ALGAE

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Aerial view of merry-go-round clay mitigation around fish cages in Yokjido Island, in the South Sea of Korea, August 21, 2008 (Photographed by Mr. JongSuk Jung)

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PREFACE

The 15th International Conference on Harmful Algae (15 ICHA) was held at the Changwon Exhibition Convention Center (CECO), Changwon, Gyeongnam, Republic of Korea, from October 29 to November 2, 2012. This conference was hosted by three organizations; the Gyeongnam Provincial Government, the National Fisheries Research & Development Institute (NFRDI), and the Korea HAB (Harmful Algal Bloom) Research Group (KORHAB). The principle aim of this conference was to clarify the role of mankind on the outbreaks of harmful algal blooms (HABs), and to determine the relevant 'must-dos' of mankind to minimize the impacts of HABs on marine ecosystems and marine industries. Therefore, the theme of the conference was "**Mankind and HABs".** There were 478 registered participants from 46 countries. To foster cooperative research and international friendship, the Gyeongnam Officials Training Institute provided 48 rooms to accommodate 77 young scientists from 22 countries at no charge. On the route to mid-day excursion to Hallyeo Marine Park in the South Sea of Korea, clay mitigation methods were demonstrated on the specially designed vessel using a suspended clay shooting gun. This illustrated the effective mitigation of fish-killing *Cochlodinium polykrikoides* blooms that can cause devastating losses to aquacultured fish along the Korean coastline.

The scientific program of the 15th ICHA focused on 17 topics that addressed a variety of global issues related to the monitoring and prediction, population dynamics, toxin chemistry, and the impact, management and mitigation of HABs. The open round table discussion on the subject "Environmentally friendly mitigation strategies" hosted local journalists and was well attended by meeting participants. A total of 395 printed abstracts representing research from 46 countries, were composed of 12 invited presentations, 139 oral talks, and 244 posters. In a departure from previous meetings, parallel sessions allowed for simultaneous presentations in specialized fields, while plenary introductory sessions featured comprehensive reviews of a range of "hot" topics.

The key-note address of "HABs in a Changing World" was presented by Dr. Donald M. Anderson of the Woods Hole Oceanographic Institution. The eight plenary lectures were: i) "Phycotoxins as Allelochemicals in Marine Foods Webs: Emerging Toxin Paradigm or New Paradox of the Plankton" by Dr. Allan D. Cembella; ii) "Growth, Feeding, and Ecological Roles of the Mixotrophic and Heterotrophic Dinoflagellates in Marine Food Webs" by Dr. Hae Jin Jeong ; iii) "Current Status and Future of HAB Occurrence and its Management in the Western Pacific Region" by Dr. Yasuwo Fukuyo ; iv) "Bloom Dynamics and Ecophysiology of the *Cochlodinium polykrikoides* with Emphasis on Korean coastal Waters" by Dr. Chang Kyu Lee ; v) "The Role of Cyanotoxins: An Open Question

to be Answered" by Dr. Sandra M. F. O. Azevedo ; vi) "Looking Back into the Future of HABs" by Dr. Barrie Dale; vii) "Green Tide in the Yellow Sea –Mechanisms and Impacts" by Dr. Mingjiang Zhou; viii) "Harm from the Benthos: Old and New Challenges for HAB Research and Management" by Dr. Adriana Zingone. Three topics, "A method to increase removal efficiency of HAB organisms by modifying clays"; "Algicidal bacteria", and "HAB mitigation strategies in Korea including environmentally friendly new initiatives" were presented during round table discussions. Of the 395 abstracts listed in the book of abstracts for the conference, 52 are presented as condensed papers in these "Proceedings of the 15th International Conference on Harmful Algae".

For a better understanding HABs in Korea, the "Harmful Algae News (UNESCO) Bulletin" No. 46, June 2012, dedicated a section to the introduction of "HAB Research and Management in Korea". The book "Korea and HABs" was distributed to all participants during the conference. In addition, a special issue of the journal Harmful Algae (Elsevier), – "Red Tides in Korea" (Vol. 30, Supplement 1, Dec. 2013) reflected trends in HABs research and management in Korea.

Members of the international and local committee served as session chairs during the conference, as well as manuscript reviewers providing assistance needed for peer-reviewed publication of these proceedings. Our deep appreciation goes to the session chairs and the patient reviewers of all manuscripts. The publication of these Proceedings was greatly facilitated and supported by the skills and dedication of Henrik Enevoldsen, IOC of UNESCO, and Karin Rengefors, webmaster of the ISSHA website.

The organizing committee and the editors of these proceedings thank all of our colleagues for their presentations which helped make the meeting a great success. Special thanks go to Dr. Allen Cembella for the summary report of the conference. We, the Local Organizing Committee and the Conference Secretariat, including three host organizations extend our gratitude to all participants for their attendance and presentations.

Hak Gyoon Kim Ph D*, Convener*

15th ICHA 2012 Korea Attendees Photographs

15th ICHA Attendees at opening ceremony, 29 October 2012, CECO, Changwon, Korea

15th ICHA Attendees at opening ceremony, 29 October 2012, CECO, Changwon, Korea

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HABs in a changing world: a perspective on harmful algal blooms, their impacts, and research and management in a dynamic era of climatic and environmental change

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Keywords: harmful algal blooms, HABs, red tides, climate change, aquaculture

Forward

More than 25 years ago, the author was asked to provide a retrospective analysis of the global status of what were then called "red tides", but are now known as "harmful algal blooms" or HABs. The occasion was an international conference in Takamatsu, Japan (Okaichi *et al.* 1989), convened at a time when algal blooms seemed to be affecting more and more countries and causing increased economic losses through a growing number of impacts, but long-term trends were anecdotal and speculative. The challenge was a significant one: to evaluate blooms of many different types of algae − some producing toxins that could affect humans, fish, shellfish, and many different marine organisms and ecosystems, some causing harm in a multitude of other ways − and then assess whether the problem was indeed "growing worse" on a global basis. The resulting paper (Anderson 1989), though highly qualitative in its approach and content, helped to ignite a scientific discussion that motivated many studies and publications, some arguing that indeed the HAB problem was growing worse as a result of pollution (e.g., Smayda 1989) or other factors such as expanded aquaculture operations or ballast water transfer of species (e.g., Hallegraeff 1993), while others contended that the "global expansion of HABs" was not accepted by all or was being exaggerated by scientists and the press. Skeptics counseled caution and argued that the increased number of toxins and impacted resources had other causes, including the simple discovery of toxins and toxic species that had always existed. It is now clear that the "global expansion" of HAB phenomena is real, due in part to our ability to better define the boundaries of the problem. However, those boundaries are not static, but continue to expand due to natural dispersal via storms or currents, as well as to effects from human influences, such as pollution, aquaculture expansion, and ballast water transport. The fact that part of this expansion is simply because of increased scientific awareness and detection capabilities should not temper our concern. The global problem of HABs is serious and much larger than we thought.

Now, at the 15th International Conference on Harmful Algae in Korea in 2012, a new challenge has been posed − this time to look forward and envision the nature of HABs, the field of HAB science, and the nature of HAB impacts and management in an era where so many features are changing rapidly due to population pressures, climatic shifts, and many other global, regional, and local forcings. Motivation for this request reflects a desire to anticipate changes that can guide research priorities, technology development, and social and commercial policies in areas that are either affected by, or that affect, HABs. The following thoughts are offered in the same manner as those written in the 1989 retrospective − as personal views offered in hopes that others will expand on these ideas and ultimately create a scholarly and comprehensive perspective on the future of HABs in a changing world.

Introduction

Over the last several decades, capabilities for research and management of HABs have grown at a rapid rate. Scientific advances have been significant in many areas, and now a large and capable research and management community exists where formerly there were only scattered individuals and programs, often working independently. Powerful new technological developments have altered the way HABs can be monitored and managed (e.g., Anderson *et al.* 2012; Scholin *et al.* 2009; Campbell *et al.* 2010). HAB problems are serious,

and in some areas of the world are growing worse, but capabilities and knowledge exist that help to minimize impacts and protect public health and marine resources as never before. This scientific and management community, and the HABs they respond to and investigate, now face a world that is changing in many ways due to population growth, pollution, and climate change, to name but a few stressors. Even the current global economic crisis can be viewed as a factor that will affect the future of HAB science and management due to reductions in funding or diversion of scientific teams to other topics, for example. Of equal importance, perhaps, perceptions of HABs are changing, affecting the behavior, needs, and priorities of the public, funding agencies, and those charged with managing these diverse phenomena. Here I explore some aspects of HABs in a changing world, looking several decades into the future in an attempt to envision the manner in which these bloom phenomena will be affected globally, and the associated challenges that the HAB research and management community will need to meet. Given space limitations and the many issues that could be addressed in this context, each can only be covered in a brief or cursory manner, but where possible, more detailed analyses or reviews of individual subject areas will be cited. Taken together, the view that emerges is one of new and exciting opportunities for research and management of HABs in our changing world, but with significant challenges as well.

Population Growth and Food Production

Population growth. The current world population of close to 7 billion is projected to reach 9.3 billion by 2050 and 10.1 billion by 2100 (United Nations, Department of Economic and Social Affairs, Population Division, 2009). This growth will not be uniform geographically, with much of the increase projected to come from high-fertility countries, 39 of which are in Africa, nine in Asia, six in Oceania and four in Latin America (Fig 1). Nearly 40% of this growing population will inhabit the coastal zone. It is well established that coastal development can lead to changes that affect some HABs − from increased pollution of the coastal zone that can stimulate some HAB species through nutrient enrichment, to alteration of coastal hydrography, nutrient dynamics, and ecosystem health and structure through the destruction of wetlands or the creation of marinas and harbors (GEOHAB 2006).

Food production and agriculture. Concurrent with this population growth will be a need to increase global food supplies by 30% by 2050. This is a massive increase, and it will have to be accomplished through expanded agriculture and aquaculture, as well as with technological advances to increase productivity in each. Agriculture will presumably account for the majority of the necessary increase in food capacity, which in turn, will undoubtedly be associated with increased fertilizer usage and thus increased loadings of nitrogen, phosphorous, and other algal nutrients to the coastal zone. Fertilizer application on land remains a major contributor to non-point nutrient pollution, and is expected to increase at an alarming rate in many regions (Seitzinger *et al.* 2010). Both industrial and developing nations are using significantly higher loadings of fertilizer in agriculture, with global N fertilizer usage increasing more than 10-fold since 1960 (Smil 2001). Looking at where fertilizers have been applied globally as a possible guide to the future, the expected pattern is non-uniform (Fig. 2; Rice and Herman 2012). The heaviest applications will once again be associated with China and India, but the United States and a number of other countries or regions such as Brazil, Canada, Southeast Asia, Russia, Africa, and Europe are expected to be major users as well.

One of the most rapidly increasing sources of nutrients to both freshwaters and the coastal zone is the atmosphere, and here again, we can expect inputs from this source to continue to grow as cities and industrial and commercial centers grow, and as more houses, cars, animal feedlots, and other sources of airborne pollution are created. In estuarine and coastal waters, it has been estimated that 20-40% of N inputs are ultimately of atmospheric origin, derived from industrial, agricultural, and urban sources (Paerl 1995).

Linkages between HABs and land-derived nutrient overenrichment have been noted within the past several decades by many workers (e.g., Smayda1989, Anderson *et al.* 2002, Glibert *et al.* 2006). Whether enhanced nutrient inputs specifically stimulate HABs is a subject of continuing debate (reviewed in Gowen *et al.* 2012), but few would contest that increased nutrient loadings can lead to increased phytoplankton growth and biomass, and in some cases, in more HABs. Going forward, coastal waters of countries with heavy fertilizer usage (Fig. 2) can thus expect higher nutrient loadings, more algal productivity, and potentially more HABs, but other countries will

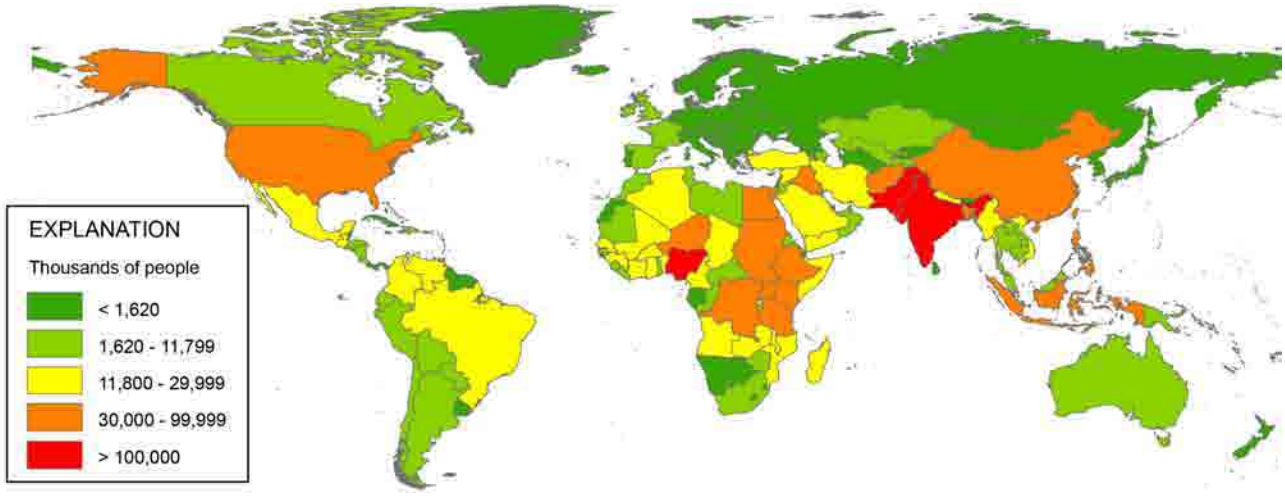

Fig. 1. Increases in the number of people by country projected for the period 2010−2050. (Rice and Herman 2012)

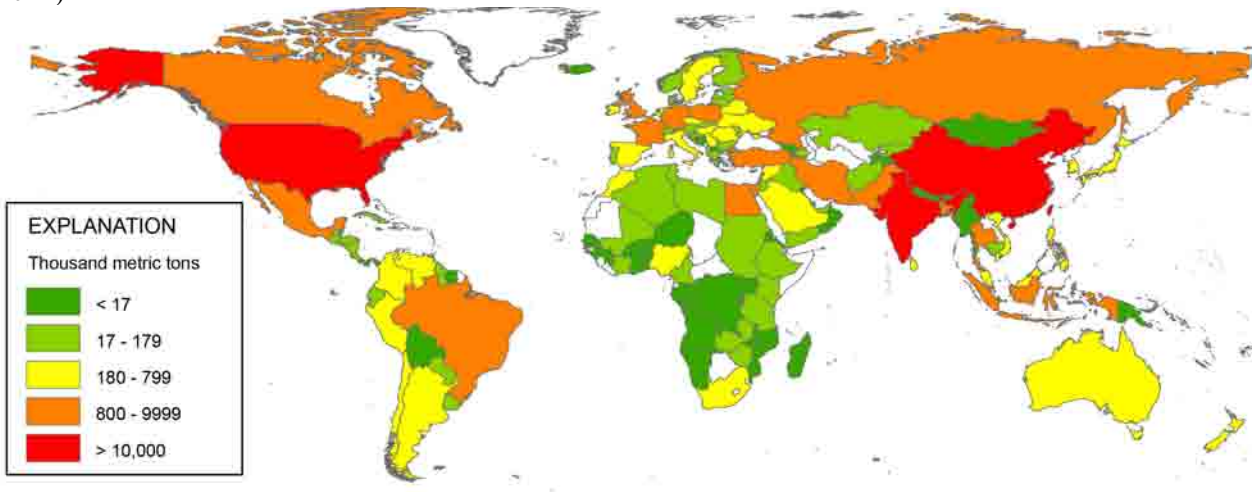

Fig. 2. Nitrogen fertilizer application by country in 2008 expressed as mass of N in thousands of metric tons. Countries with no reported data are shown as white. (Rice and Herman 2012)

be affected as well, even those with modest increases in nutrient loadings from agriculture. Note also that the algal proliferations resulting from these nutrient inputs are not always immediately evident as toxic or destructive HABs, but can instead cumulatively lead to major downstream effects known as 'dead zones' - areas where the dissolved oxygen levels are so low that marine life cannot be sustained (other than micro-organisms). Since the 1960s, the number of dead zones in coastal waters has doubled every decade (Diaz and Rosenberg 2008), and given the expanding population and food production, it seems inevitable that this aspect of HAB impacts will continue to grow through time.

Capture fisheries and aquaculture. One might hope that a significant portion of the world's growing need for food could derive from wild fisheries, but this is decidedly not the case. Overall global capture fisheries production continues to remain stable at about 90 million tons (FAO 2012). Furthermore, most stocks remain fully exploited or over-exploited. As of 2006, FAO reports that 52% of the world's commercial fish stocks are fully exploited, 19% are over-exploited, with 8% "significantly depleted" and 1% slowly recovering (FAO 2012). Only 20% of stocks are considered under-exploited or moderately exploited. Of the world's 15 major fishing regions, productivity has fallen over the past few years in all but four. The alarming state of capture fisheries

prompted FAO (2012) to warn: "the maximum wild capture fishery potential from the world's oceans has probably been reached."

While capture fisheries production remains stable, aquaculture production has been expanding rapidly. Aquaculture remains one of the fastest-growing animal food-producing sectors, and in the next decade, total production from both capture and aquaculture will exceed that of beef, pork or poultry. In the last three decades, for example, world food fish production from aquaculture has expanded almost 12 times, at an average annual rate of 8.8% (FAO 2012). China continues to account for the largest share of farmed marine and freshwater species, with 70% of the total volume and over 50% of the total value. Other major producers in Asia are India, Vietnam, Indonesia, Bangladesh, Thailand, Myanmar, the Philippines, and Japan.

Aquaculture can thus contribute substantially to the global need for increased food production, but it also represents another source of HAB nutrients, provided as feed or fertilizer, and modulated by the biological transformations occurring in these high biomass systems. Cultured fish retain only a fraction of their food − the rest decomposes in the water column or settles to the bottom and decomposes; either way, the nutrients released from this decomposition can stimulate phytoplankton growth. Even molluscan mariculture can be a cause for concern, as the long-held view that such production is always environmentally favorable because the natural water-clearing effects of filter feeding shellfish is now being questioned in certain situations, particularly those with heavy stocking densities (Bouwman *et al.* 2013). The concern is that, because of their low assimilation efficiencies, molluscs can become point sources of regenerated nutrients, acting as pumps that transform the nutrients in algal biomass into dissolved and particulate forms. Benthic regeneration of the accumulated feces and decomposing feed can thus be a significant and sustained source of nutrients in such systems. In their review, Bouwman *et al.* (2013) examined several scenarios of future aquaculture development, and estimated that nutrients from aquaculture operations of all types will increase up to six-fold by 2050, and more importantly, that this increase may exceed the nutrient assimilative capacity in those parts of the world where aquaculture is rapidly growing.

To reduce nutrient effects from these expanding operations, production may have to be moved to areas that are well flushed or that are located in deeper, more dynamic offshore waters. This is happening to some extent in the U.S. and some other countries, where fish farming operations have shifted from easily accessible but poorly flushed bays and coves to areas with much stronger currents − resulting in a significant reduction in particulate and dissolved nutrient buildup, and consequently, reduced planktonic and benthic impacts. In contrast, most fish farms in developing countries are located in shallow, easily accessible bays where nutrients can accumulate and stimulate algal blooms. The effect can be worsened when aquaculture sites are located in wetlands (e.g., salt marshes or mangrove swamps) that otherwise would serve as a sink rather than a source of nutrients to the system.

The takeaway messages from these predictions of population growth and the need for expanded food production are several: 1) eutrophication-related HABs are not going to decrease in the near future, but are likely to increase on a global basis; 2) the distribution of nutrient-enhanced HABs will not be uniform, as many countries with both rapid population growth and increased agriculture and aquaculture production (e.g., China, India, Africa) will continue to be "hot spots" for such outbreaks, unless major policy changes are instituted that lead to more efficient application of fertilizers or to the optimal location and operation of aquaculture facilities; and 3) aquaculture operations will continue to increase dramatically going forward, providing high quality food for a growing population, but also contributing to the HAB problem, through nutrient enrichment in poorly flushed areas, as well as the placement of susceptible resources in areas where HABs can cause problems, through both toxicity and high biomass effects.

These scenarios are not speculative − there is already ample evidence that these impacts are occurring. For example, China has experienced rapid increases in eutrophication, mariculture, and other population-related pressures in the coastal zone, and this has been accompanied by an equally dramatic increase in the frequency and diversity of HABs, as well as changes in the species composition of harmful algae (Zhou and Zhu 2006). Virtually all of the main HAB species that we are familiar with worldwide have been observed over the last decade or two in Chinese waters, some detected for the first time (Lu *et al.* 2014). As might be expected given the heavy nutrient inputs to coastal waters from anthropogenic sources, large-scale, high-biomass blooms (those covering more than 1,000

km^2) have been increasing dramatically along the coast. *Prorocentrum donghaiense* has been a recurrent bloom species in the East China Sea for more than ten years, and other high biomass bloom formers include *Phaeocystis globosa*, *Cochlodinium geminatum*, and *Karlodinium veneficum*. Brown tides caused by *Aureococcus anophagefferens* have recently occurred in the Bohai Sea. Just 20 years ago, there were only a handful of HAB scientists in China, and only a few recognized species of concern. Clearly times have changed, as China has developed one of the largest national communities of HAB researchers and managers anywhere, and given the trends noted above in population growth, fertilizer usage, and mariculture expansion, this country and others that are developing in similar ways may need to expand their HAB research and management capabilities even further.

Species dispersal, range expansion, and new toxins

From the foregoing, it is apparent that for some parts of the world at least, the HAB problem will remain the same, or more likely, worsen with the combined pressures of a growing world population, food production, aquaculture development, and coastal development. Superimposed on this type of HAB expansion will be the emergence of new species or impacts. Although many HAB species are already widespread, some continue to appear in new locations, as has been the case with *Cochlodinium polykrikoides* in recent years (Kudela and Gobler 2012). *Aureococcus anophagefferens*, the tiny brown tide organism that was formerly restricted to the northeastern US and South Africa, is now causing massive blooms along the coast of China (Zhang *et al.* 2012), and another brown tide species − *Aureoumbra lagunensis* − has expanded its range from a single area in the Gulf of Mexico to Florida (Gobler *et al.* 2013), and more recently to Cuba (Koch *et al.* 2014). *Ostreopsis* is another emerging problem − formerly of concern as a benthic dinoflagellate potentially linked to ciguatera fish poisoning, but more recently shown to be the source of toxic aerosols that cause respiratory problems and illness among beach goers. As stated by Rhodes (2011), the distribution of *Ostreopsis* has expanded markedly in the last decade, associated illnesses have also increased, and these trends are likely to continue.

These are but a few examples of the continuing expansion of HAB species globally, and there seems little doubt that these types of reports will continue for many years. One could argue, however, that we will frequently be documenting the dispersal of HAB species that we already know about, with fewer and fewer new species or toxins being described. This reflects the maturity of the HAB field, as well as the global distribution of many HAB species. However, as discussed below, species that are not traditionally considered to be harmful are being identified as HAB species as new societal activities or resources are affected in negative ways by algal blooms.

With respect to toxins, the pace of discovery of new toxins has slowed from the days when saxitoxin and brevetoxin were known, but dinophysistoxins, pectenotoxins, domoic acid, yessotoxins, spirolides, azaspiracids, palytoxins, and others were yet to be identified or described. It has been more than a decade since the characterization of the azaspiracids (James *et al.* 2003), the last major toxin associated with a new and previously unknown acute human toxicity syndrome (azaspiracid shellfish poisoning − AZP), and about five years since euglenophycins were described (Zimba *et al.* 2010). This trend suggests that the 'golden age of discovery' of new classes of phycotoxins causing human toxin syndromes may be behind us (Anderson *et al.* 2012). There will, however, still be new toxin discoveries going forward, but these will presumably occur less frequently than in the past. Meanwhile, HAB chemists will remain busy with the development of new analytical technologies and approaches to measure known toxins, identification of derivatives and metabolites of the known toxins, and characterization of mechanisms of biotransformation and bioaccumulation. One compilation (P. Hess, pers. comm.) lists 444 toxins in the major HAB toxin families, with 322 known chemical structures. These include 10 compounds in the domoic acid family, 18 saxitoxins, 60 okadaic acids, 18 azaspiracids, and 7 microcystins, to name but a few. In addition to these, there are numerous metabolites − nearly 40 additional compounds in the saxitoxin family alone (Weiss *et al.* 2010). Many HAB toxin metabolites are not fully characterized, so it will take many years to document their potency, structure, and public health significance. The latter is of particular importance, as managers are already struggling with the need to monitor multiple toxins in seafood or drinking water, and adding more compounds to that list will be problematic for many. It is important for resource managers to

know that there are numerous derivatives and metabolites potentially present, but the realities of measuring so many closely related compounds argues for simplification of the monitoring process (through functional rather than analytical assays, or LC/MS methods that allow concurrent detection of many toxins, for example). ELISA assays are currently available for most of the major HAB toxins, and this brings toxin detection capabilities to many who lack expensive and complex instrumentation for direct chemical analysis. Fishermen can now test shellfish and other commercial products themselves, opening up access to resources that are otherwise restricted. A prime example of the value of these kits is the re-opening of the offshore surf clam and ocean quahog industry on Georges Bank, which had been closed to harvest for over 20 years due to the threat of PSP toxins. Development of an Onboard Screening, Dockside Testing Protocol based on saxitoxin ELISA kits has resulted in the sustained harvest of millions of dollars of offshore shellfish in that region (DeGrasse *et al.* 2014), and it is likely that the approach will be used to maximize the harvest of offshore shellfish in other areas as well.

One major gap in our current toolbox for HAB toxin detection is the lack of a reliable kit-based assay for ciguatoxins. Ciguatera fish poisoning (CFP) remains the most serious of all HAB human poisoning syndromes globally, with estimates of 50,000 or more poisonings annually. There is a clear need for inexpensive and widely-deployable ciguatoxin detection capabilities that can protect populations from poisoning, and allow consumption of reef fish that are presently avoided due to the risk of CFP. Although the technical challenges to developing an antibody with sufficient specificity to the large ciguatoxin molecule are significant, new approaches such as single-domain antibodies may provide this much-needed capability going forward.

Changing perceptions of HABs

One expectation for the future is that some countries or regions may begin to reevaluate the way that algal blooms are viewed, and in particular, which species are considered harmful. The term HAB has always been broad, as it was intended to include toxic blooms as well as those that cause harm in other, diverse ways. Despite the long list of HAB impacts that are well known and recurrent throughout the world, new impacts will emerge going forward, and with that will come the designation of new harmful species. One current example is with desalination plants. The expansion of HABs due to pollution, coastal development, and other factors, is occurring at a time when there is also an increase in the construction of desalination plants to produce drinking water. There are currently more than 14,000 desalination plants in more than 150 countries worldwide, and the desalination market is forecast to grow by 12% per year. The intersection of these plants with nearshore HABs is inevitable. Recent research suggests that algal toxins are effectively removed by desalination processes and pretreatments (e.g., Laycock *et al.* 2012, Dixon *et al.* 2011), so the larger concerns relate to algal biomass. It is now clear that algal species can produce organics that pass through ultrafilters and other pretreatment processes, forming gels or polymers (e.g., transparent exocellular compounds or TEPS; Berman 2012.) or extracellular polymeric substances (EPS; Flemming and Wingender, 2001) that are either the direct cause of fouling or that serve as a nutrient source for the microbial communities that foul the membranes. These compounds can be seriously disruptive, particularly to those plants that use reverse osmosis (RO) to produce fresh water. One example is the bloom of *Cochlodinium polykrikoides* in the Arabian Gulf and Sea of Oman in 2008/2009 that affected a large number of RO desalination plants, closing some for as long as two months (Richlen *et al.* 2010). Since economic considerations are leading to a huge expansion in RO desalination plants compared to those that use flash evaporation or other thermal processes, we can expect many more impacts of HABs on desalination facilities than have been recorded thus far. It is also very likely that species that are not generally considered harmful to other sectors of society will be harmful to these plants because they produce disproportionally large amounts of dissolved organic materials. Eventually, a list of species that are prolific producers of harmful organic compounds (that are not toxins) will be generated and used by desalination plant operators to plan mitigation strategies.

Another aspect of the changing perception of HABs stems from the fact that many are very well understood and managed in certain countries. This in turn can lead to a shift in research funding to other areas − either to other HAB problems or syndromes, or to unrelated areas. One example is

in Japan, where the management of PSP and DSP is so effective that human illnesses are rare. As a result, funding agencies do not feel it is worth investing in research on the fundamental science of those topics; in effect, they are not "problems" any more. Now many HAB scientists in Japan are shifting their research to topic areas where major unknowns remain – to ciguatera fish poisoning, for example (Y. Fukuyo, pers. comm.).

A related shift in some countries is from fundamental research on physiology, ecology, and genetics to topics directly applicable to management, such as bloom control or impact mitigation. In one sense, this is a reflection of the maturity of the field, and the progress made in our efforts to understand the fundamental mechanisms underlying HABs and their impacts. However, this trend towards more practical and pragmatic HAB research is not necessarilly a wise approach. In the U.S., for example, the HAB community has long argued that the most effective national HAB program is one that sustains the full spectrum of research, including the Ecology and Oceanography of HABs (the ECOHAB program; Anderson 1995), Monitoring and Event Response for HABs (http://www.cop.noaa.gov/stressors/extremeevents/hab/current/fact-merhab.aspx) and Prevention, Control, and Mitigation of HABs (PCMHAB; Dortch *et al.* 2008). However, in a sign of what many other countries are already experiencing or will experience, the funding emphasis within these national programs has changed in recent years due to pressures from mission-oriented agencies with clear mandates for practical outcomes such as improved forecasts or bloom control. The challenge in the U.S. and elsewhere is thus to maintain an emphasis on fundamental HAB science and resist the temptation to shift resources to solely address more practical aspects.

Yet another interesting change in the perception of what is harmful comes from countries that are heavily dependent on their coastal waters for aquaculture and capture fisheries, particularly those with very dense operations such as in China, Japan, Korea, and other Asian countries. Here we are seeing a distinction being made between HABs and FABs or "favorable algal blooms" as countries are recognizing that phytoplankton biomass needs to be at a relatively high level to support such operations. In this sense, algal blooms, even dense, high biomass ones, can be considered beneficial, and thus efforts to reduce pollution or other nutrient inputs as a general bloom mitigation strategy may not be supported by certain sectors of society, such as fishermen. An interesting example comes from the Inland Sea of Japan, an area frequently used as an example of the manner in which reductions in pollution can lead to corresponding reductions in algal blooms, including those that are destructive to fisheries and aquaculture. Between 1965 and 1976, the number of red tide outbreaks (high biomass blooms) increased seven-fold in the Inland Sea, in parallel with the increase in industrial production and chemical oxygen demand (COD) from domestic and industrial wastes (Okaichi 1997). In 1973, Japanese authorities instituted the Seto Inland Sea Law to reduce COD loadings to half of the 1974 levels over a three-year period. The number of red tides began to decrease in 1977, eventually falling to less than 30% of the peak frequency, which had been in excess of 300 blooms per year. However, in the years since that legislation, fisheries productivity has also decreased in the region, leading to requests by the fishing industry for pollution controls to be relaxed, in the hopes that this will lead to more algal productivity and blooms, and foster enhanced fisheries production (Y. Fukuyo, pers. comm.). Implicit in this type of request is the acceptance of an occasional destructive HAB event in return for generally enhanced productivity and higher fishery yields the remainder of the time. One wonders if this view of favorable, high-biomass algal blooms will become more prevalent as countries and agencies worldwide are under increased pressure to maximize coastal fisheries productivity to feed their growing populations.

HAB prevention, control, and mitigation

The subdiscipline of HAB prevention, control, and mitigation (PCM) is diverse, as it covers a wide array of strategies that can reduce the impacts of HABs (Boesch *et al.* 1997). It is obviously preferable to prevent HABs in the first place rather than combating their impacts, so an array of strategies have been formulated through time, including shellfish toxin monitoring programs, controls on nutrient inputs to water bodies, and early detection and forecasting of blooms. Many believe that some of the global expansion in HAB incidence is linked to increased pollution of the coastal ocean, particularly by plant nutrients (e.g., Smayda 1989, Anderson *et al.* 2002, GEOHAB 2006). Indeed, there are few other causes that could be responsible for the scale and timing of the observed increases in HABs over

the last several decades. As a result, conscientious pursuit of goals for pollution reductions, including excess nutrients, could well prevent HABs in some locations. Such strategies are typically longer-term in nature, however, due to the buildup of a reservoir of nutrients in bottom sediments and adjacent soils in many areas. Careful assessment and precaution against species introductions via ballast water and aquaculture-related activities also can be effective preventative strategies.

Mitigation strategies are based on the acceptance that HABs are going to occur, and therefore strategies for reducing impacts are needed. Example strategies include depuration of toxic shellfish, towing of fish pens to clear waters, harvesting restrictions on toxic shellfish, or early harvesting of threatened fisheries products. The procedures used to keep contaminated fisheries resources from the market have been largely successful when appropriately applied (Anderson *et al.* 2001). However, in order to contend with more frequent and diverse risks from HABs in an era of declining governmental resources to support labor-intensive monitoring, more sophisticated and reliable detection methods are required, in addition to the immediate expansion of simple methods, such as ELISA toxin kits, or networks of volunteer phytoplankton observers (Hall 1999), for example. These new technologies are rapidly appearing, as discussed below, and will continue to facilitate efforts to mitigate HAB impacts, even in areas with recurrent and serious HABs. Individuals consuming seafood and medical professionals seeing patients also need to be better informed about the risks, and responsible public education and communication should therefore receive increased attention. Progress has been excellent in this important element of HAB impact mitigation, but education and outreach efforts need to be sustained and further strengthened.

The one PCM area where development has been particularly slow and uneven on a global basis is in bloom control or suppression. In 1997, the author wrote a commentary in *Nature* (Anderson 1997) that attempted to explain why HAB science had such a striking lack of research emphasis and thus progress in this important area. At the time, an international conference on HABs in Vigo, Spain (1997) had one scientific presentation on direct bloom control, out of 400 papers or posters from 58 countries. Multiple explanations were offered, including the lack of targeted funding opportunities specifically for bloom control and impact mitigation, the complexity of the dynamic, three-dimensional ocean and the ecosystems in which marine HABs occur, and the relative difficulty of getting favorable peer reviews for proposals on controversial bloom control strategies in competition with fundamental studies of bloom dynamics or ecophysiology. Now, nearly 20 years later, the situation has improved, but progress is still much slower than in other areas of HAB science. At the recent International Conference on Harmful Algae (ICHA) in Korea in 2012, 30 papers on bloom control were presented, out of 380 total. This may seem like major progress, but most of these studies originate from only a few countries (Korea, China, and Japan for marine HABs), and the total also includes freshwater HAB control studies, which were not included in the 1997 Vigo conference. The challenges to controlling HABs in freshwater lakes and rivers are certainly significant, but are less complex than in the ocean, and there is wider public and management acceptance of such environmental manipulations and mitigation efforts given the long history of water-body treatment for drinking water supplies. It thus seems likely that major HAB control efforts will advance faster in freshwater systems than in the marine environment.

It also seems inevitable that there will be a stronger and stronger push for more progress on HAB control, as this is what the general public and most stakeholders feel should be a major target and outcome of HAB research. In the U.S., legislation has been passed that includes authorization of a funding program that focuses specifically on prevention, control, and mitigation, as many politicians believe that this should be the top priority for the funding they provide. As discussed above, it has sometimes been a battle to convince these legislators to sustain funding for fundamental HAB science, given their desire for practical approaches to control and management. Given the trends noted above, the recognition that politicians and agencies are becoming increasingly pragmatic about HAB research priorities, and the widespread human manipulation of the coastal zone for food and commerce, we can expect that bloom control research efforts will expand in the future. In the U.S., for example, the PCMHAB program was established as a targeted program in which all funding applications fall within the same topic area of practical PCM strategies, such that unfair comparisons between practical versus fundamental science are not made during the peer review process.

Practical control strategies tend to be controversial, and often speculative or unproven. Perhaps even more important is the requirement that all funded PCMHAB projects include a Transition Advisory Committee consisting of stakeholders, managers, and others who will benefit from, or be affected by, the proposed mitigation strategy. By including these individuals in project meetings from the outset, environmental and social objections can be addressed at an early stage, and the transition to full application made less confrontational and controversial. Despite this positive perspective, it seems likely that bloom control efforts will continue to focus on specific, high-value resources in countries that are best able to appease environmental interests, leaving other countries with only prevention and mitigation in their PCM arsenals.

Molecular biology and genetics

Recent development and application of advanced technologies in genomics, transcriptomics, and proteomics has already provided deep insights into the ecology, physiology, taxonomy, and toxicology of HABs, and this trend will surely continue. Noteworthy contributions to HAB science have been made in many areas, one of which is the taxonomic and phylogenetic reclassification of a number of HAB species. For example, the dinoflagellate genera *Gymnodinium* and *Gyrodinium* were traditionally separated on the basis of strict morphology (girdle displacement), but molecular phylogenetic approaches led to the creation of the genera *Karenia* (10+ spp. now known.), *Karlodinium* (8 spp.) and *Takayama* (6 spp.) (Daugbjerg *et al.* 2000, de Salas *et al.* 2008). Similar reclassifications have now been made for *Gambierdiscus* (Litaker *et al.* 2009) and more recently, the *tamarense* complex of *Alexandrium* (John *et al.* in press).

Another major area of progress has been in the elucidation of biosynthetic pathways and identification of genes involved in processes such as toxin production (Kellmann *et al.* 2008, Stucken *et al.* 2010) through transcriptomics and gene expression profiling. As the genomes and transcriptomes of more HAB species are described, discoveries of key physiological processes like these will continue, deepening our understanding of the mechanisms underlying bloom dynamics, toxicity, and general impacts. Likewise, molecular tools have revealed genetic heterogeneity at multiple levels during HABs, including within individual bloom populations. Indeed, the demonstration that HABs are assemblages of multiple strains or genotypes that will differentially adapt to a changing environment is critical to our understanding of bloom dynamics, including responses to climate change and other anticipated forcings.

Transcriptome analysis only applies to expression at the gene transcriptional level, but many key genes are post-translationally modified, particularly in dinoflagellates. One way around this constraint is through analysis of the proteome, a cell's complement of proteins. This has only been applied to HAB taxa to a limited extent (e.g., Chan *et al.* 2005, Nosenko *et al.* 2006, Young *et al.* 2009, Wang *et al.* 2012) but there is great potential in this technology, and as more protein data are made available in databases, this should be an area of significant growth.

A related field of endeavor – metabolomics – also has the potential for great advancement in the field, but as with proteomics, the study of the composition of low-molecular weight metabolites within cells has yet to be applied to HAB research in a concerted fashion. There are no apparent major technological impediments, and the metabolomics approach has already been successfully applied to the dinoflagellate endosymbiont *Symbiodinium* (reviewed by Gordon and Leggatt 2010). Among HAB species, metabolic fingerprinting of *K. brevis* extracts by mass spectrometry was not successful in identifying specific compound(s) responsible for allelopathy (Prince *et al.* 2008). This frontier field is likely to be more fruitful when additional comprehensive spectral libraries become available, and as structural-functional relationships of the metabolites are better defined.

Looking forward once again, it is clear that significant research advances will be achieved through the individual use of the "omic" technologies, but that major advances will also benefit from their combined use as datasets and analytical capabilities expand in each.

New technologies for in situ HAB cell and toxin detection

Anderson *et al.* (2001) reviewed the different approaches adopted by countries and commercial enterprises worldwide to monitor and manage HABs in coastal waters. There are, however, many challenges associated with these activities due to the complexity and diversity of HAB phenomena. Resource managers and regulatory officials must deal with multiple toxins and multiple toxic algal species, multiple toxic fisheries resources, and large-

and small-scale HAB events that occur intermittently. Many new approaches to HAB cell detection and bloom monitoring have been developed, and many more are anticipated in the future.

Among the new approaches to species-specific detection and enumeration are optical instruments, such as the autonomous underwater vehicle (AUV) called the Brevebuster, which enumerates cells using optical features unique to *K. brevis*, the Florida red tide organism (Richardson and Pinckney 2004). The Imaging FlowCytobot (IFCB) is another powerful new optical tool – it is essentially an underwater flow cytometer that uses a range of optical features in an automated classification approach to photograph, enumerate, and identify cells at the genus, and sometimes species level (Campbell *et al.* 2010).

Yet another approach to improved cell detection involves the development of species- or strain-specific molecular "probes" that can label HAB cells of interest so they can be detected visually, electronically, or chemically. This line of research has been a hallmark of the HAB field because of the need for species-specific measurements. Progress has been rapid, and probes and assays of multiple types are available for many HAB species, with more being added every year. These developments have reached the stage where the new molecular counting methods are routinely employed in major research and monitoring programs.

These probe methods also opened the door to remote, subsurface, near real-time detection of specific HAB taxa. One instrument that provides these capabilities for in situ HAB cell and toxin detection is the Environmental Sample Processor (ESP; Scholin *et al.* 2009). The ESP autonomously collects discrete water samples from the ocean subsurface, concentrates microorganisms (particulates), and automates application of molecular probes to identify specific microorganisms and their gene products. Capabilities have also been developed for antibody-based detection of two HAB toxin families – domoic acid (Doucette *et al.* 2009) and saxitoxin (G. Doucette unpub. data). There are now 18 ESPs in use around the globe, some devoted to HAB studies, but others used for investigations of microbial communities, larvae, and other components of planktonic ecosystems (Scholin *et al.* 2009). Looking forward, we can anticipate a time when the ESP, IFCB, Brevebuster, and other in situ autonomous instruments are included as operational assets in ocean observing systems – arrays of moored and mobile instruments that can collect and transmit data continuously from remote locations to shore-based scientists and managers. We are still in the early stages of a shift from at-sea research to the use of robots and observing infrastructure to collect data, and this trend will surely continue to shape the nature of HAB monitoring and management in the future. Just as networks of meteorological stations and numerical models of atmospheric dynamics greatly improved our ability to provide accurate forecasts of weather events, ocean observatories and their associated numerical models of ocean dynamics have the potential to document long-term patterns and changes in the sea, to detect infrequent HAB events that previously went unobserved, and to make predictions or forecasts about these and other phenomena that directly affect human populations and marine ecosystems (Anderson *et al.* 2008).

HAB modeling and forecasts

Technological advances have expanded our capabilities for research and monitoring of HABs, but the blooms will always be undersampled because of the large space and time scales over which they occur (McGillicuddy *et al.* 2010b). This has led to the development of models of various types, and more recently to forecasts. There are those in the HAB field who feel that it is not possible to model or forecast complex HABs in the dynamic environment of open coastal waters (or large freshwater systems for that matter), yet progress has been significant and the results compelling, with much more progress expected going forward, and many research teams pursuing this important goal.

One example of an innovative and useful empirical model is that of Raine *et al.* (2010) who described a chain of observable events that lead to blooms of *Dinophysis acuminata* in Bantry Bay, Ireland. The authors defined a single index that quantifies these patterns and used that to evaluate past outbreaks, and to predict new ones.

Numerical models with varying levels of sophistication have also been developed. Some are three-dimensional physical models that resolve hydrography, into which HAB cells are introduced as passive particles (e.g., Velo-Suarez *et al.* 2010). A similar approach is used in a HAB forecasting system developed for *K. brevis* blooms in the Gulf of Mexico (Stumpf *et al.* 2009). Blooms are

detected and defined using ocean color satellite images, and bloom transport is predicted using hydrographic modeling with the HAB cells treated as passive particles. This approach is also being taken for cyanobacterial bloom forecasts in Lake Erie (Wynne *et al.* 2011). The next step in sophistication and complexity has been to couple a detailed biological submodel to a hydrographic model, as has been done for *Alexandrium* blooms in the Gulf of Maine region in the U.S. (McGillicuddy *et al.* 2005). This model has demonstrated good skill at reproducing observations (He *et al.* 2008) and has been heavily used for hindcasts (i.e., looking at past events to understand underlying mechanisms; He *et al.* 2008, Li *et al.* 2009). It is also being used to issue weekly nowcasts and forecasts (looking forward three or four days), and even seasonal or annual forecasts (McGillicuddy *et al.* 2011).

Despite significant advances in recent years, there are many aspects of the HAB modeling effort that need to be improved. Regional models need to be developed for many parts of the world, but this can be facilitated by the use of computer code and algorithms developed for other areas or other HABs. A major area for advancement will benefit from the collection of in situ HAB data on a real-time basis that can be assimilated into the models to improve accuracy, much as is done with meteorological sensor networks and weather forecasts.

A realistic vision for the future is therefore that of arrays of moored or mobile instruments with sensors for HAB cells and their toxins generating data for the numerical models that provide forecasts with sufficient accuracy to be of use by managers, fishermen, the general public, and other stakeholders.

Climate change

Over the past several decades, many new or unexpected HAB phenomena have been attributed to eutrophication or ballast water introductions, but now, climate change is increasingly invoked as a potential factor (Hallegraeff 2010). This view reflects undeniable changes in atmospheric CO_2 concentrations, rising global temperatures, melting of glaciers and ice caps, and changing storm, rainfall, stratification, and acidification patterns. This aspect of HAB research and policy (reviewed in Hallegraeff 2010) is in its infancy, as publications are few, and those that do exist tend to focus on single environmental factors (e.g., CO_2, temperature increase, stratification), single biological properties (photosynthesis, toxicity, nutrient uptake), or individual species or strains. Complex physical and ecosystem interactions are rarely considered, and most time series of HABs are not of sufficient duration to permit realistic extrapolations. Another major constraint is the general lack of regional physical-biological models of HAB systems that can be utilized in forecast mode to explore future climate change scenarios. Relatively few of these exist worldwide (McGillicuddy 2010a), and where they do, they are not yet coupled to larger, ecosystem-level models or to global climate models. Although some regional ecosystem models exist, we need to augment the output to include parameters that give us more detail for HAB projections. In other words, one can simulate HAB growth and population development under different climate scenarios, but this will be relatively meaningless unless one also knows what these changes will do to co-occurring phytoplankton, zooplankton, predators on zooplankton, and other elements of the food chain that can directly affect the HABs being simulated. Compounding the problem is the relative coarse resolution and accuracy of the global climate models into which the higher resolution local or regional HAB modeling must be nested.

Prediction of the impact of global climate change on HABs is thus fraught with uncertainties, yet many HAB researchers are being asked by resource managers, the general public, and other stakeholders to make those types of forecasts. There is no question that HAB research will have climate as a major focal area for the foreseeable future, and that there will be range extensions and contractions of key HAB species, yet our ability to make definitive statements about expected changes and impacts will continue to be limited for many years.

As described above, multi-factorial experiments are necessary, yet are exceedingly time-consuming and difficult to undertake in a meaningful way. In this regard, we need to take maximum advantage of "natural" experiments. Very few long-term records exist of HABs at any single locality, and as a rule we need at least 30 consecutive years before trends can realistically be detected (e.g., Anderson *et al.* 2014). Much can be learned, however, from long-term data sets for phytoplankton in general, such as those from the Continuous Plankton Recorder (CPR) (e.g., Burkhill and Reid 2010) or from short-term phytoplankton community responses to

El Niño-Southern Oscillation (ENSO) and North Atlantic Oscillation (NAO) episodes or other large-scale meteorological and oceanographic events. In the latter context, one of the best ways to study climate impacts may well be to take advantage of weather fluctuations that mimic future climate change scenarios. This might, for example, be a major regional warming event, or an intrusion of a water mass with different properties into an area, as has occurred in a number of areas subject to HABs in recent years (e.g., McGillicuddy *et al.* 2011). If "rapid response" funding is made available, it might be possible to take advantage of these weather perturbations to study entire eco- and hydrographic systems under a common forcing. Fig. 3 shows the extraordinary scale of a warming event in the North Atlantic in 2012. The anomaly stretched over thousands of kilometers, with temperatures as high as 3 °C above the 30-year mean. Studying a natural event of this type may be more informative than running multiple laboratory experiments on individual organisms and then trying to extrapolate those results to predict the responses of a complex ecosystem. With natural weather fluctuations, entire systems are responding to the change, and if these are well documented, a great deal of information can be obtained, and a more complete story developed.

A re-examination of the phytoplankton fossil record using increasingly sophisticated geochemical tools (e.g., Dale 2001) is also warranted, as climate trends of the magnitude that we will be experiencing in the next 100 years have happened before, albeit at a much slower pace and starting from a cooler baseline than present (IPCC 2008). Past episodes of climate change over long periods of geological and evolutionary history allowed organisms to adapt to their changing environment, and much can be learned from these ancient patterns.

There are clearly a number of challenges facing HAB scientists as they attempt to determine whether the local or regional phenomena that they study are being affected by climate change. This is obviously a long-term endeavor, but without the proper tools and approaches, trends will be difficult or impossible to definitively identify, and mechanisms will be obscure, making it all the more difficult to convince state, federal, and local agencies that changes are indeed occurring and require some level of response or adaptation. Strong efforts should be made to establish long-term datasets of not only HAB cell

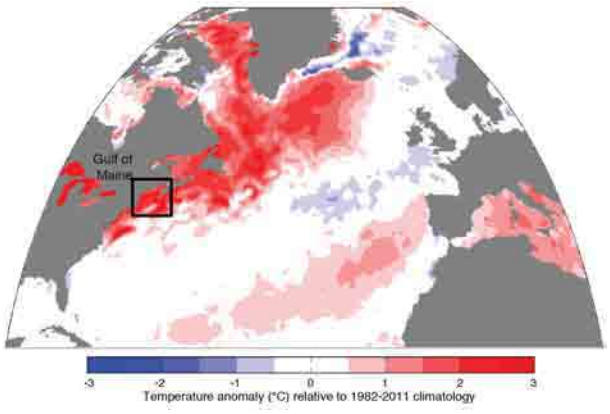

Fig. 3. Mean sea surface temperature (SST) anomalies for the Northwest Atlantic and the Gulf of Maine for June-August 2012. The anomoly values are relative to the 1982-2011 climatology. (from Mills *et al*. 2013)

abundance or toxicity thorough time, but also a range of other parameters that affect HAB abundance. These would include nutrient concentrations, hydrographic features, and regional meteorology, as well as the abundance and distribution of competing phytoplankton species, potential grazers, parasites, or viruses. We need several decades of data of this type to begin to reveal trends that are more than mere fluctuations in a noisy, long-term signal. Realistically, however, establishing and maintaining long-term datasets of this type is extraordinarily expensive and challenging, particularly if efforts are made to obtain a diverse array of data to place the HAB observations in context.

To meet this overarching goal for long-term data, a number of possible programs or approaches might be considered. These would include: a) utilization of data from state or federal monitoring programs; although usually limited to a single parameter (e.g., shellfish toxicity), these data can provide continuity through time and broad spatial coverage, as well as information on the onset, termination, and intensity of outbreaks (e.g., Anderson *et al.* 2014); b) identification of key locations within individual countries or regions where sufficient data can be collected to provide the necessary perspectives; it will not be possible to determine climate change effects on all HABs, but if a few key representative groups or habitats are characterized, it may be possible to formulate generalizations that apply more broadly to HABs of many types; and c) development and utilization of new sensors and instruments that can collect long-term data in an

automated, high-frequency, and sustained fashion. These could be deployed through existing ocean observing networks, but many of those are not in locations where recurrent HABs occur, so HAB-specific deployments will also be needed.

Summary

Clearly, there are many challenges facing HAB scientists and managers as well as the HAB species themselves in this rapidly changing world. A growing world population and the need to provide food for 30% more people in the next 40 years will lead to increased nutrient enrichment of coastal waters. As a result, we can expect more HABs in some areas as well as increased impacts due to the expansion of aquaculture operations in affected regions, and perhaps even the direct stimulation of HABs by these operations. These expanded impacts will be most significant in developing areas of the world that are already struggling with growing HAB problems – areas like China, southeast Asia, India, and Africa. In some areas, pressures to reduce nutrients are going to conflict with the need for enhanced coastal productivity in support of dense aquaculture (the favorable algal bloom or FAB concept). It is also clear that there will be other ramifications of the changing perception of HABs by resource managers and funding agencies, potentially shifting funding away from certain topics (e.g., fundamental ecology and physiology) and into others (e.g., prevention, control, and mitigation). Driven by the need for more accurate yet less-expensive monitoring data, improvements in technologies for cell and toxin detection will continue at a rapid pace, as will the efforts to incorporate some of these technologies into autonomous biosensors that can augment monitoring programs and provide valuable, real-time data to improve detection and forecasting efforts. Molecular biology and 'omic' technologies will continue to provide novel insights into HAB taxonomy, evolution, cell physiology, and gene function. With respect to climate change, other than stating that there will be range extensions and contractions for HAB species, little else can be predicted about future changes due to the current lack of long-term data sets and the complexities within the environments and ecosystems where HABs occur. Even the most advanced HAB models are far from the stage where long-term forecasts are possible that can reflect these complexities and incorporate future climate projections in a way that is realistic. Incremental progress in forecasting is needed, however, and should not be discouraged because of its high uncertainty at present. Valuable information about the resilience of HAB species and communities can be gleaned from weather or climate variations that mimic future climate change scenarios, so every effort should be made to take advantage of these as they occur. Overall, there will continue to be many new challenges in HAB research and management, but the tools, technologies, and skilled personnel are in place to minimize impacts and protect public health and marine resources as never before.

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Looking back into the future of harmful algal blooms and HAB research[1]

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Abstract

Scientists are increasingly expected to predict the effects of global change on harmful algal blooms (HABs), with no long-term records to show the effects of previous change. This may be compared to a person trying to work out a sensible travel plan for where to go, without first knowing where they are or where they are coming from. The often stated aims of HAB research are to better understand HABs in order to predict and if possible mitigate their harmful effects. This requires a better understanding of the ecology of HAB species, but these only differ from the rest of the plankton by their harmful effects on humans, so the need is for better understanding of plankton ecology as such: establishing which species live where and when in order to address how and why they do so. Available plankton records are often inadequate for this and the scientific community should consider a shift in emphasis back to more basic ecology by maintaining and further developing sound long-term plankton monitoring to find out what is actually happening in the plankton. Such information would help relate much of the current research to natural plankton populations; without such information, attempts at prediction are premature and unrealistic. The sedimentary record of dinoflagellates, from up to 40% of coastal species that produce fossilisable cysts, provides long-term evidence of natural variation through time suggesting ecological response to environmental change. This includes some HAB-species, but for other important species we are reliant on long-term plankton records.

Keywords: Harmful algal blooms, dinoflagellate cysts, long-term plankton records

Introduction – the need to first "look back"

Today, basic research is regarded by many as a direct service industry for society where scientists are expected to predict future threats to health, wealth and well-being of the people. However, sound prediction first requires a basic understanding of the current state of affairs, which in turn relies to some extent on a perspective drawing on knowledge of past situations. There is a useful analogy with travel: If you do not know where you are coming from, it is often difficult to know where you are and almost impossible to estimate where you are headed. In this respect, the time perspective is one of the most important, but often least understood considerations in the natural sciences. Thus, scientists attempting to predict future harmful algal blooms (HABs) could well begin by "looking back" to see if available time-series data are adequate for the task?

[1)] "Looking back" at past HABs

The term "harmful algal bloom" includes a wide range of phenomena, the full extent of which cannot be addressed within the space available here. Any species or combinations of species within the plankton may be capable of causing harm and disruption to humans if they reach sufficient concentrations. This is not only achieved by increased growth. In some cases species may be concentrated simply by natural processes of wind, currents, and water density gradients (many of the most dramatic illustrations of dense "red tides" in the literature are in fact of this type of "bloom"). Otherwise-harmless species have regularly contributed in this way to oxygen deficiency from breakdown of their biomass, resulting in deaths of fish and other animals of interest to humans. Also, such high cell concentrations have sometimes caused problems by clogging up the workings of fishing-gear, water filters for desalination plants, power

1) Summary of plenary lecture at ICHA15, Korea

plants, etc. However, much of the public concern regarding blooms has concentrated on the toxin-producing species that directly threaten the health, wealth and well-being of humans. There are two main ways of assessing these and other past HABs: estimated economic losses (not considered further here), and cell concentrations recorded in the plankton. How reliable are available plankton records for assessing past HABs? Cell counts documenting the concentration of HAB-producing species in the plankton provide some of the most important primary data for investigating HABs. However, while the long established sampling methods for counting are simple, the process is labor-intensive, and the needs for adequate sampling of water masses subjected to transport and mixing are often poorly met. The extent of the difficulties encountered is shown here in an example from the record of the fish-killing species *Karenia mikimotoi* from the Sherkin Island Marine Station phytoplankton monitoring program (Figs. 1and 2, and Table 1). This unique program, ongoing since 1978, is based on much greater sampling density than most other reported programs, particularly with respect to coverage within the water column. The record of *K. mikimotoi* at Sherkin Island will be dealt with more comprehensively in a forthcoming publication (Dale and Murphy, in prep.), but Figs 1 and 2, and Table 1 serve to show the great variation in cell concentrations of just one species encountered within one region on one day. The long-term record since 1978 shows similar degrees of variation on the scale of weeks to years. This casts doubt on the accuracy of previous HAB records elsewhere based on similar counts from far fewer samples: Just how many cells were responsible for these previously recorded "blooms"? Even with the unusually high sample coverage in the Sherkin Island record it is not possible to identify "normal" concentrations of any given species at a given time, exposing flaws in the earlier definitions of HABs based on "unusual" amounts of a species. "Looking back" into the Sherkin Island record suggests that many other available plankton records may have too few samples to reveal the true variation present at any given time. Similarly, almost all available records cover relatively few years and are probably inadequate to reveal long-term natural variation in HABs. The inadequacies of long-term phytoplankton records and HABs have prompted the search for other complementary data. Two main sources have proven useful so far: The Continuous Plankton

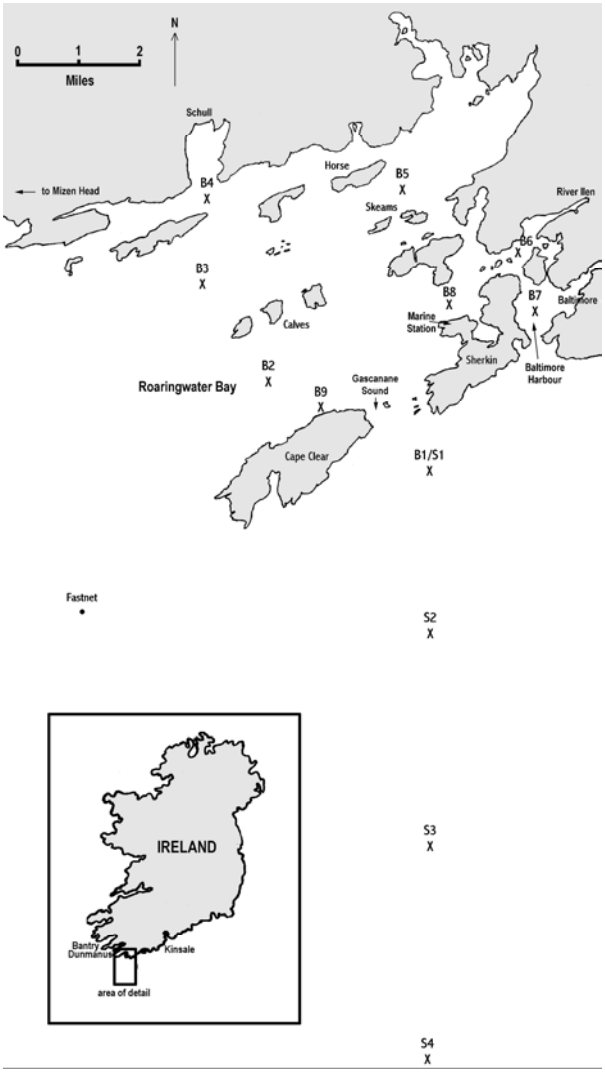

Fig. 1. Map showing stations routinely sampled for the Sherkin Island plankton monitoring program. Inset shows location within southern Ireland.

Recorder survey, though designed to monitor for zooplankton, has produced useful data for some larger HAB organisms in parts of the North Sea and North Atlantic (Warner and Hays 1994), and microfossils in bottom sediments offer possibilities for long-term records for those species preserved (Dale et al.2006).

Dinoflagellate resting cysts can provide complementary data

The potential role of microfossils to at least partially extend the record of plankton further back in time has been documented (Martin 2000), and will not be repeated here. Suffice it to say that

Table 1. Count data in cells/liter from all stations, on July 11th, 2010.

Karenia mikimotoi **- cells/l**

Date	Station	0m	2.5m	5m	7.5m	10m	15m	20m	25m	30m	50m
11.07.2010	S4	100	600	8800	-	13600	39200	53100	28300	500	0
11.07.2010	S3	2400	6000	8600	-	42100	50700	40500	49500	200	800
11.07.2010	S2	2000	19900	43500	-	59300	11800	400	300	800	0
11.07.2010	B1/S1	39000	40900	23900	-	11900	6900	4100	10200	6000	2700
11.07.2010	B2	24200	14200	21800	-	1600	3300	800	500	200	-
11.07.2010	B3	4400	6300	10700	-	3200	1200	1400	300	600	-
11.07.2010	B4	1500	3300	3200	-	300	0	0	-	-	-
11.07.2010	B5	4200	1400	400	-	200	-	-	-	-	-
11.07.2010	B6	2000	1500	300	-	-	-	-	-	-	-
11.07.2010	B7	6200	20200	12800	-	-	-	-	-	-	-
11.07.2010	B8	2800	1800	4900	3600	8800	-	-	-	-	-
11.07.2010	B9	9800	2600	900	-	-	-	-	-	-	-

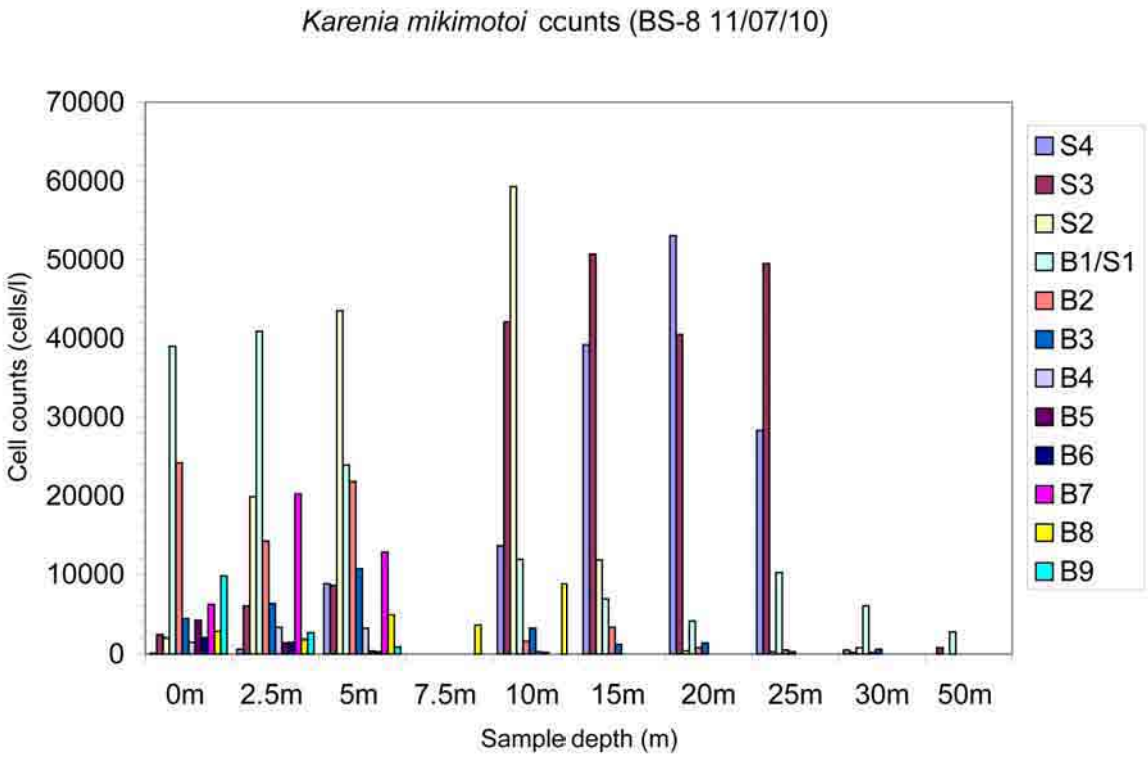

Figure 2. Data from plankton counts from all stations, on July 11th, 2010.

utilizing the microfossil group of my choice, dinoflagellate cysts, we have been able to identify environmental signals from the global distribution of cysts in recent marine sediments today, model these, and successfully apply these models to interpret paleoenvironments from sedimentary sequences on time-scales of tens, thousands and millions of years (Dale & Dale 2002; Dale et al. 2005).

The robustness of these environmental signals strongly suggests that the cysts are reflecting fundamental aspects of dinoflagellate ecology, and this could be of direct interest for HAB research. Many HABs are caused by dinoflagellates, and much HAB research therefore seeks to better understand the ecology of these species. However, "HAB species" are not an ecological group; they are only distinguished from the rest of the plankton by their disturbance to humans, so we need to understand plankton ecology as such in order to maintain a balanced perspective. Jeong et al. (2010) showed a

glimpse of the largely unknown extent of complexity of food webs within the plankton, just one reminder that the field of plankton ecology is still in its infancy and needs all the input it can obtain in order to progress. Why should the cyst record provide ecological information that seems not to have been seen from plankton records? The motile stages of dinoflagellates that make up a sizeable portion of the plankton in many regions and cause some of the most damaging HABs are highly biodegradable, leaving no distinctive morphological record in bottom sediments. In contrast, resting cysts are protected by a more resistant cell wall that in many species persists into the sediments after death to leave a more permanent fossil record. The cyst approach is seriously limited, however by the fact that not all HAB species form cysts, and not all species that do form permanently resistant cysts (see discussion in Dale et al., 2006). Nevertheless, the cyst record provides ecological information for a significant number of dinoflagellate species (up to 40% of coastal assemblages, Dale 1976), largely due to its integration of the short-term variation in plankton. The cyst assemblage from one sample collected from the surface of bottom sediments represents an integrated record of up to decades of variation in the plankton, depending on rates of sedimentation, and suites of samples from dated core sediments provide an integrated record of up to thousands of years. For cyst-forming species this has allowed us to compare the integrated cyst record from surface sediments with available integrated hydrographic data to reveal ecological signals. The strong implication from this is that it would take many decades of detailed plankton records such as that of Sherkin Island to realistically explore phytoplankton ecology. Long-term cyst records from sediment cores are beginning to reveal evidence of increased dinoflagellate "blooms" associated with past climatic oscillations and cultural eutrophication (Dale et al. 2006)

"Looking back" at HAB research

The author has argued for many years that ICHA conferences should include a session to assess the state of HAB research. A few brief comments on my view of HAB research over the past forty years are included here in an attempt to prompt others to contribute their views to "where we are coming from, where we are, and where we are going with HAB research". The often stated aims of HAB research are to better understand HABs in order to predict their occurrence, and if possible mitigate their harmful effects. This involves gaining a better understanding of when, where and how a given species blooms – in other words, of its ecology. In my opinion, much of the research so far has been hampered by the misguided concept that the ecology of a species may be studied more or less in isolation from its natural occurrence within the population of other species in the plankton. In many cases, local HABs have been caused by one particular species, at least for a number of years, and research has attempted to concentrate on this one target species: counts of its occurrence in the plankton (sometimes only the target species) over relatively few years, and lab experiments to culture the species and determine its "special" environmental requirements. Funding pressure may have driven this approach, since it is easier to understand a concentrated effort directed towards the problem species, but this has generally failed to seriously improve our understanding of HABs or deliver the desired prediction capability. As already noted, convincing long-term plankton records may be needed to reveal enough of phytoplankton ecology to better understand HABs, but this has not been attempted on the required scale so far by the research community. Early plankton ecologists such as Trygve Braarud understood the need for both field observations and appropriate laboratory experiments (Smayda 1993). Unfortunately, his vision of establishing a long-term plankton monitoring program in Oslo could not be realized (Braarud pers. comm. 1976) due to developments in marine science affecting most research groups from the 1960s onwards. These included an attitude that "old-fashioned" science based on counting plankton samples under the microscope must give way to more technical science, and that phytoplankton could be estimated by chlorophyll measurements in the "bigger picture" of things. The limitations from this approach may be seen from the recent compilation of the ecology of harmful algae (Granéli and Turner 2006). This shows great progress in many diverse aspects related to plankton ecology, but often without a unifying sense of how this relates directly to population biology, a need suggested by several of the authors. This limited progress in basic ecological research may have helped fuel the other major efforts which have adapted technological progress in other fields to develop new methodology for HAB studies. Great

progress has been made in identifying the main toxins affecting some HABs, and molecular genetics is providing probes for identifying the species, though we are still far from a molecular version that can replace the traditional morphologically-based phytoplankton taxonomy. These new "tools" certainly help protect the public from some of the worst effects of HABs, with short-term monitoring programs now better able to detect the build-up of harmful species and toxins in the food web. The extent to which they improve our understanding of HAB phenomena as such, or help us predict HABs, remains to be seen. During the past few years a major attempt has been made to use modeling to predict HABs in the Gulf of Maine and elsewhere. The models utilized were based on relatively sparse observations in space and time of concentrations of the one target species in seawater during blooms and measured concentrations of its cysts in bottom sediments at one time in the previous fall. Initial cyst abundance was suggested as the most important factor determining the intensity and extent of the blooms (Li et al. , 2009). Cyst concentrations in the "seedbed" were presumed to remain constant from fall to spring (despite possibilities for disturbances by bioturbation, storms and fisheries), and there seems to be no measured confirmation of cysts actually excysting to initiate the blooms. To date these models have not produced convincing predictions.

Looking into the future of HABs and HAB research

Non-toxic HABs may be expected to increase in future with increased impact on the marine environment from a rapidly increasing human population. There will be more gear and installations in the sea for HAB species to disrupt, and greater possibilities for cultural eutrophication to increase algal biomass. However, much of the interest in future blooms concerns toxin-producing species that directly threaten the health, wealth and well-being of humans. Understanding the ecology of these species and working towards eventual prediction of where and when they bloom represents the greatest challenge to future HAB research. There has been much speculation and discussion within the research community as to whether these HABs are increasing globally. There have certainly been cases of apparent increases in localities such as the Seto Inland Sea of Japan and Hong Kong harbor, where eutrophication has been identified as a major contributor (Glibert et al. 2005). Nevertheless, the issue of global increase is complicated by several factors. No standard definition of "blooms" has been applied, so global comparisons of different datasets remain questionable. Also, aquaculture installations have greatly increased globally, and this, together with a general increase in awareness from the many more people now monitoring for HABs globally, has lead to increasing numbers of reports of HABs in the past three decades. The earlier suggestion of a developing global epidemic of HABs (Smayda 1990) has received little supportive evidence. Nevertheless, many reports and research proposals continue to suggest that there is a global increase. However, the extent to which HABs really are increasing or there are simply more reports from increased attention to the problem, remains largely unresolved, and the reasons for any supposed general increase remain hypothetical. A main aim of future research should be to document possible increased HAB activity and investigate its causes. Although here again, only long-term records will allow any observed increase to be viewed from a perspective of natural variation.

Expected effects on HABs of a changing world?

It may prove to be unfortunate that the public concept of a future changing world is at present almost exclusively dominated by fears of global warming. Before addressing this important topic, it is therefore appropriate to mention other factors most likely to affect future blooms. One prediction that seems certain is that the global human population will greatly increase in the near future, including a disproportionate increase within coastal regions. This poses a threat of increased impact on the marine environment in a variety of ways that may affect the frequency and intensity of HABs; a few examples are suggested here. Future increased nutrient levels in the ocean and freshwater bodies are a main concern. The coastal zone in particular is vulnerable, serving as the recipient of human waste and pollution while at the same time providing increased food from fisheries, and aquaculture, and freshwater from desalination plants. This has clearly resulted in increasing problems with HABs in the past (Gilbert et al. 2005), demonstrating the need for responsible resource management practices to minimize negative effects in future. The need for more human food production will inevitably promote the continued increase in aquaculture worldwide. Since feed is the limiting factor for aquaculture, this in turn will

increase pressure on deepwater fisheries to provide fishmeal supplemented increasingly by plankton feed (krill from Antarctic waters is already beginning to be exploited). The mounting concern for science is no longer simply considerations of top-down effects from over- fishing – the whole system may be threatened with disruption. A much more comprehensive understanding of plankton ecology would be needed to predict the consequences of this for HABs. In general, increasing human impact on the ocean will have comparable effects to those experienced from the longer record of terrestrial impacts. Feral species will be introduced, as they already have been, to new regions (ships' ballast water, commercial shellfish seed, etc.), including HAB-producers. Similarly, some local habitats will be drastically altered, for example by coastal building projects such as new harbors. This may well create seedbeds of cysts accumulating in artificially sheltered waters along otherwise open coasts with far less possibility for cyst accumulation. This has a potential for increasing HABs locally.

Predicting HABs and global warming

The theory of human-induced global warming (IPCC2007) is widely accepted by many scientists and politicians, though challenged by others (Humlum et al. 2011; 2013). This issue has in common with HABs that it arouses fear among the global public who then demand predictions of possible consequences. As in other fields of natural science, HAB-researchers are expected to predict the effects on their studied organisms, and this exercise exposes real limits to our understanding of HABs (Dale et al. 2006). One prediction regarding global warming seems relatively sound: climate has varied in the past and therefore may be expected to do so in future. This natural variation comprises cycles of alternatively warmer and colder oscillations expressed on timescales of millions of years (interglacial/glacial) to hundreds of years (e.g. Medieval Climatic Optimum (MCO)/ Little Ice Age (LIA)). It also includes regional climatic oscillations on the order of tens of years (e.g. the North Atlantic Oscillation (NAO), and similar regional phenomena around the World affecting short-term climate). Temperature records of hundreds of years would be needed to pick up signals from the longer-term temperature trends on the scale of the MCO and LIA. Decadal records would reflect more influence from local short-term oscillations. This creates difficulties for climatologists trying to distinguish human influence from the naturally-warming climate emerging from the "Little Ice Age".

The few long-term plankton records at best cover only a few tens of years, comparable to perhaps just one of these short-term regional temperature oscillations. Thus, the available time series plankton data are inadequate for establishing species' responses to natural climate variation and therefore offer no sound basis for predicting effects of climate change. Ironically, proponents of human-induced global warming face similar problems, since actual global time-series temperature measurements may prove inadequate for distinguishing possible human impact from natural global warming.

General considerations for future research

HAB research faces an increasing dilemma shared by other fields of natural science: humans face problems in their interactions with the natural world, they fund science to investigate these, and in return they understandably expect answers to practical problems. The dilemma arises from the fact that our basic understanding of the enormous complexity in the natural world often lags far behind the ability to provide the answers. Nevertheless, in areas of science such as HAB research that originate from human problems, it is difficult to justify a purely theoretical approach seemingly remote from the public need for risk assessments relating to health and food production.

In the case of HABs, the practical problems involve protecting public health from toxic food and managing sustainable food production with minimal economic loss from the blooms. As argued here, this requires a greater understanding of plankton ecology: finding out which species live where, and when, in order to ask the important questions of how and why they do so. There is now a great deal of information on which species live where globally, but too little information on when they do so, often restricted only to when they cause problems. However, information from when a species does not bloom at a given site often may be just as important for understanding why it blooms when it does.

Here, I have tried to underscore the need for addressing this time perspective. I believe there is a need to shift some of the research emphasis from technical developments and premature attempts at modeling back to more basic ecology. The sedimentary record

of dinoflagellate cysts shows significant variations in species composition on timescales of decades to thousands of years. Restricted as this is, from only cyst-forming species, it most probably represents a small glimpse of much more natural variation in the plankton than is seen from most available plankton records. HABs must be viewed as part of this variation. A strong case can be made for suggesting that understanding long-term variation is crucial to better understanding HABs. In which case the question may be raised as to what extent we can claim to be studying bloom phenomena *without* including long-term plankton monitoring (Dale 2005). Funding agencies may resist long-term commitment of funds, but the HAB scientific community should unite behind a collective international effort to secure continuation of the few existing long-term plankton monitoring programs, particularly those which count all species; and where possible new programs should be initiated. If such a monitoring program had been in place in Massachusetts in the years leading up to the famous first ever recorded HABs there in 1972 and subsequent years, for example, we would now have the possibility of investigating the causes, but these instead remain a mystery, even after many years of research since. Such a dataset would be invaluable, too, for relating all the subsequent research projects generated by this event to the population dynamics of the actual system.

Even a brief consideration such as this of where we are in HAB research strongly suggests that prospects for predicting HABs will remain slight for a long time into the future. A parallel may be drawn to the extreme difficulties experienced in long-term weather forecasting, due to the complex variation in the physical elements involved. Weather is only one of many physical factors affecting HABs, added to which is the still poorly understood biological complexity of plankton ecology. Meanwhile, great progress is being made by local monitoring programs, particularly in commercially important regions. While unable to provide long-term prediction of HABs, this has succeeded in protecting public health for many years now, and should continue to improve its effectiveness as more experience is gained and shared globally. Cyst surveys offer a potential for helping to address some of the practical problems expected to arise in future. For example, mapping of cysts in sediments along the Alaskan coast would help identify possible areas with lower risk of HABs as a first step towards opening up a region of vast shellfish resources; and monitoring for cysts in sediments in new marine building projects such as artificial harbors will help reveal possibilities for new HAB seed-beds in newly accumulating sediments. The value of this type of preliminary cyst survey was demonstrated by a project we carried out recently for the Environmental Agency of the United Arab Emirates. 59 cyst types were recorded from selected samples from both sides of the Arabian Peninsula, including some HAB species not previously recorded in the plankton. Surveys like this provide valuable baseline reference data, and the agency is considering including cyst surveys as a part of its HAB monitoring program as human development of the coastal zone progresses. Cyst concentrations in all the sediment samples proved to be unusually low, leading to a new hypothesis of natural mitigation. A well-tried mitigation technique demonstrated from Korea involves spraying clay particles into the water to sediment out and dilute HAB species from the plankton. Along the coast of the Arabian Peninsula regular sandstorms from the desert may be spreading sand into the sea, in effect severely diluting cyst concentrations in bottom sediments. Lower cyst concentrations would presumably reduce initiation of new blooms from the "seedbed" - mitigation of the initiation, rather than the full plankton bloom. This may not help understand the causes of massive blooms of *Cochlodinium,* thought to have originated offshore and experienced in the region one to two years prior to sampling for cysts. However, such blooms were not repeated in subsequent years, and since only a few questionable cysts of this type were recorded, it may explain why no apparent seedbeds were established for this species in the region afterwards that could have promoted new blooms.

The geological prospects for contributing to HAB research increased dramatically recently when new techniques for studying sediment fabric in minute detail revealed monospecific layers of fossil dinoflagellates in repetitive marine sequences indicating seasonal cyclicity – the nearest evidence yet of ancient "blooms". And the latest developments in molecular biology are probing even further back in time with the discovery of comparable genes for the production of saxitoxin, the most potent dinoflagellate toxin, both in dinoflagellates and cyanophyte bacteria (Stüken et al. 2011) – likely reflecting shared time for these two ancient groups or their predecessors in the primeval ocean.

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Harm from the benthos: old and new challenges for HAB research and management

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Abstract

The recent expansion of *Ostreopsis* and *Gambierdiscus* blooms across coastal oceans has turned attention to the benthos and posed new challenges to scientists and managers. Several aspects concerning blooms of benthic microalgae are indeed distinct as compared to planktonic ones, requiring new research approaches, adequate sampling methods and tailored physiological and morphological investigations. Cryptic and pseudo-cryptic diversity has been revealed in both *Gambierdiscus* and *Ostreopsis*, in parallel with the recognition of the functional consequences of this diversity. A specific adaptation to benthic life is the production of mucous, at times in the form of a thick layer, but whether the mucous is also related to mixotrophic nutrition is not clear. Peculiar to Benthic Harmful Algal Blooms (BHABs) are their ways of impact, including relationships with the co-occurring marine fauna. The toxins produced by benthic species can generate a range of impacts encompassing damages to the benthic fauna, fish and shellfish poisoning syndromes and aerosol or seawater-borne human syndromes. Environmental alterations under the pressure of climate variations and ocean acidification could affect the diffusion and impacts of benthic harmful blooms, possibly through changes in local hydrography and their effects on the benthic biota.

Keywords: Benthic Harmful Algal Blooms, *Ostreopsis*, *Gambierdiscus*

Introduction

Human health problems caused by Benthic Harmful Algal Blooms (BHABs) are well known in tropical and subtropical areas, where *Gambierdiscus toxicus* and a number of allied species cause the long recognized and ever increasing syndrome named ciguatera (Skinner *et al.* 2011). Even more impressive expansion and intensification of BHABs, caused by species of the genus *Ostreopsis*, have affected the coasts of temperate and warm-temperate areas such as the Mediterranean Sea, the Atlantic, West Pacific and New Zealand (Fig. 1).
The increasing interest for benthic HABs is shown by the exponential increase in papers published on this issue over the last couple of years, most of which focusing on *Ostreopsis* species. The wide distribution of *Ostreopsis* species and the remarkable toxicity of the palytoxin-like substances that they produce certainly deserve as much attention as the one given to *Gambierdiscus*. In this overview, some aspects of organisms causing BHABs are summarized, with no intention to be exhaustive. The aim is to highlight a number of research issues that are peculiar to these species and discuss the need to assess their ecology to envision current and future scenarios of their distribution.

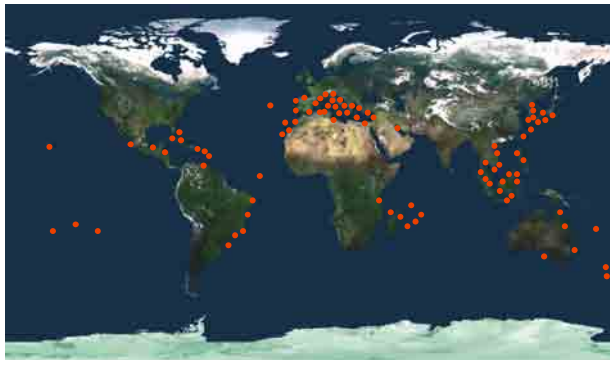

Fig. 1. Worldwide distribution of *Ostreopsis* spp. as of 2012.

Gambierdiscus and *Ostreopsis*

The knowledge of the ecology and impact of benthic harmful species is hampered by the peculiar aspects of these species in relation to their life style. While benthic ecologists have rarely considered microalgae in their research, most specialists of microalgal ecology have restrained their studies to planktonic organisms. However, benthic microalgal ecology presents several aspects which markedly differ

from those offered by the planktonic life (Fraga *et al.* 2012). The information which has accumulated about *Gambierdiscus* and *Ostreopsis* species over the last years has been compiled in some comprehensive reviews (e.g. Rhodes *et al.* 2010, Parsons *et al.* 2012). A recently established core project of the IOC-SCOR GEOHAB program (Zingone *et al.* 2012) has resulted in a report (Berdalet *et al*. 2012) which provides a synthesis of the available information as well as of scientific and technological efforts needed.

Based on these reviews, both analogies and differences are traceable between *Gambierdiscus* and *Ostreopsis* species. Analogies concern the benthic nature of these organisms, including the production of a mucous matrix, as a means of attachment to the substrate, and a generally high spatial heterogeneity. The latter is difficult to address, as several distinct sampling methods have been used, resulting in the lack of a common metric and, at times, scarcely comparable results. Transitions between the benthic and pelagic life are frequent in both cases. Differences are also remarkable between *Gambierdiscus* and *Ostreopsis*, for example in temporal distribution - more or less constant in the former and highly seasonal in the latter - the only partially overlapping geographic ranges, and in the much higher abundances generally attained by *Ostreopsis* outbursts.

Conflicting results are often shown by the comparison of different studies, e.g. for light, temperature, salinity, nutrients, substrate and wave energy preferences, as well as for seasonality. In the case of *Gambierdiscus*, this might be explained by the fact that for a long time several species have been considered as a single taxonomic entity, which probably is true also for *Ostreopsis*.

Taxonomic variations

Gambierdiscus taxonomy has undergone considerable changes over the last years, leading to a definition of several cryptic or pseudocryptic species that may have different distribution, physiological characteristics and toxicity (Litaker *et al.* 2009; Kibler *et al*. 2012). For *Ostreopsis*, the taxonomic status of the more widely distributed taxa, namely *O. ovata* species-complex and *O. siamensis*, is still to be defined, but also in this case cryptic species have been identified with different toxicity and physiological characteristic (Sato *et al.* 2011).

Interestingly, the genetic variability in the *O. ovata* species-complex is much higher along the Japanese coasts as compared to the Mediterranean-Atlantic area (Penna *et al.* 2012; Sato *et al.* 2011). This situation indicates a relatively recent radiation of the species in the latter area and, given the lack of hydrographic links between the two regions, a possible man-mediated transport of some lineages from the Pacific to the Atlantic-Mediterranean region, although it is impossible to establish when this occurred. A route for the translocation of benthic dinoflagellates is through aquacultured shellfish, which may harbor macroalgae and their epiphyte on their shell. Trade exchange of seafood has been intensive and unregulated for many years. While even such variable markers as rDNA ITS fail to show genetic differentiation within the Atlantic-Mediterranean *Ostreopsis* cf. *ovata*, geographic differences may exist at the population level, as shown by recent AFLP-based studies (Italiano *et al.*, this volume).

Adaptations to benthic life

Life in the benthos implies peculiar adaptations which can be reflected in both physiological and morphological traits. A clear example is the flattened shape of benthic gonyaulacoid dinoflagellates as compared to related species living in the plankton.

A peculiarity of both *Gambierdiscus* and *Ostreopsis* is the formation of abundant mucous material which connects the cells to the substrate altering its nature, a clear case of ecosystem engineering. Cells can either be embedded in the mucous matrix or have less tight relationships, which allow them to easily migrate from benthos to plankton. In the case of *Ostreopsis*, both mucocysts and trichocysts contribute to form at times very thick mucous layers, which eventually may detach from the substrate and float, conferring an unpleasant aspect to seawater. Unique ultrastructural features have been identified in benthic gonyaulacoids (Besada *et al.* 1982) including a channel where mucocysts apparently convey mucous before discharging it externally through a mouth-like aperture, the ventral opening (Escalera *et al*. 2014). This latter structure was previously interpreted to be involved in prey ingestion (Faust 1998), but evidence for mixotrophy in *Ostreopsis* species is still elusive. *Gambierdicus* also shows interesting and unique ultrastructural features (Besada *et al.* 1982; Durand et al. 1986), including mucocysts, pseudonuclear vesicles and acid phosphatase (PAS) bodies, the latter possibly involved in intracellular digestion. How these

structures relate to cell attachment to the substrate and to possible mixotrophic nutrition is still to be assessed.

A dual relationship with benthic invertebrates

The trophic relationships involved in the transfer of toxins across the food-web differ between *Gambierdiscus* and *Ostreopsis.* While ciguatera is mediated by herbivorous fish, risks deriving from palytoxin-like substances concern a wide range of marine animals, including bivalve mollusks, cephalopods, sea urchins and fish (Aligizaki *et al.* 2011). In addition to the risks caused by contaminated sea-food ingestion, *Ostreopsis* species can also cause health problems by direct contact of seawater and through aerosols (Fig. 2).

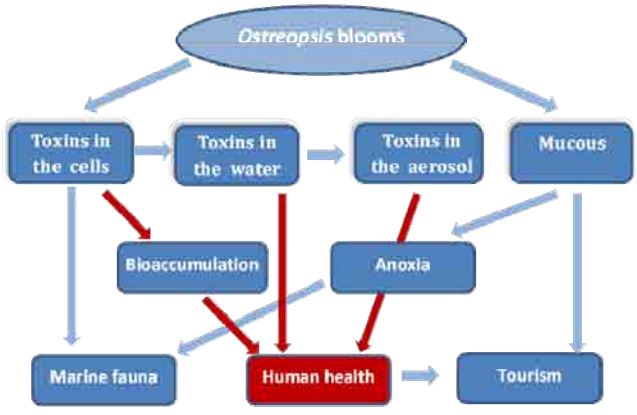

Fig. 2. Impact routes of *Ostreopsis* spp. blooms. In red, those riskful for human health.

While at times marine benthic invertebrates are affected by *Ostreopsis* blooms, e.g. showing malformations, loss of appendages, and even death (Shears and Ross 2009), in several cases both invertebrates and fish can accumulate the toxin. These dual effects are also seen in laboratory experiments conducted on the bivalve *Mytilus galloprovincialis*. The animals can actively filter *Ostreopsis* and accumulate toxins only when exposed to low cell concentrations, showing instead pathological symptoms at higher concentrations (unpublished results). Similarly, the sea urchin *Paracentrotus lividus* can feed on macroalgae colonized by *Ostreopsis* with no apparent harm, but show concentration-dependent pathological symptoms and even death when exposed to cultures of the same species.

BHABs and global change

BHABs have long been reported from tropical and subtropical areas. Accordingly, the recent increase of their impacts has been related to temperature variations affecting our planet over the last decades. This association is however not always obvious. Under predictable higher temperatures, *Gambierdiscus* ranges may show an expansion towards higher latitude, but also a contraction in warmer tropical areas (Llewellyn 2010; Tester *et al.* 2010). Distribution data demonstrate that *Ostreopsis* species are neither restrained to warm waters (Fig. 1), nor to the warmest season in the temperate zone. For example, in the mid and north Adriatic Sea the blooms occur in late-summer autumn, when sea-water temperature can range between 17 and 22° C (Totti *et al.* 2010; Mangialajo *et al.* 2011). In fact laboratory experiments show that *Ostreopsis* species can grow over a relatively wide range of temperature values, at times with some contradictory results (Granéli *et al.* 2011; Scalco *et al.* 2012) probably due to strain variability. In general, the simple extrapolation of laboratory results is risky, as temperature changes can be dampened by physiological plasticity, which can hardly be revealed by short-term experiments, or through evolutionary adaptation, which can occur in unicellular organisms over a relatively short time. Moreover, the impact of temperature variations on species distribution and abundance in the sea is mediated by hydrography (e.g. wave intensity and currents) which may lead to counterintuitive and hardly predictable consequences. The impact of ocean acidification on the distribution of marine organisms may also occur through complex mechanisms. The demise of coral reefs, as a consequence of ocean acidification and increased temperature, can lead to a shift towards macroalgal-dominated systems, which are more prone to BHABs. Direct effects are instead more unlikely. Indeed, at a site with volcanic CO_2 emissions of the Ischia Island (Mediterranean Sea) *Ostreopsis* cf. *ovata* was recorded at bloom concentrations at pH values similar or even lower than those predicted for the 2100 coastal ocean (Di Cioccio *et al.*, this volume). By contrast, *Ostreopsis* abundance differed by an order of magnitude at adjacent sites, in relation to their exposure level, which again highlights the relevance of hydrographic features to the intensity of BHABs.

Concluding remarks

Knowledge on species causing BHABs is still in its infancy. The actual diversity of these organisms has only recently been addressed properly, while the functional consequences of this higher diversity are just starting to be acknowledged. Most studies have been restrained to a limited number of geographic areas. Hence it is likely that an even higher diversity will be discovered in *Gambierdiscus* and *Ostreopsis* upon more extensive studies, as it has occurred for several planktonic species.

The biology of benthic microalgae reveals specific ways of life and adaptations. Dedicated studies may hence highlight new structures and functions in these species. Research on benthic dinoflagellates as new model organisms can indeed benefit the whole field of microbial ecology. For example, population genetic studies may be facilitated in benthic as compared to planktonic species, which would help to shed light on mechanisms of speciation in dinoflagellates.

The peculiarities of benthic harmful species with respect to the planktonic ones call for specific, standardized methods for both ecological and physiological studies.

Trophic pathways and relationship with the co-occurring fauna and flora are also highly specific in the case of benthic microalgae, and deserve attention due to their possible impact on human health. These aspects call for interdisciplinary research soundly rooted in the knowledge acquired in the field of benthic ecology.

Prediction of future trends requires a better appraisal of the physiological and evolutionary adaptation capabilities of benthic dinoflagellates and consideration of both direct and indirect effects of climate variations.

The latter aspects would benefit from a closer collaboration with coastal oceanographers, considering the relevance of hydrographic conditions in the coastal zone to the development of BHABs and to the modulation of their impact.

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Regional events and trends, biogeography, and novel and alien species

Evaluation of *Pseudo-nitzschia* spp. in a tropical bay of the Mexican Pacific

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Abstract

The Acapulco Bay, located in Mexican tropical Pacific, shows high phytoplankton species richness, especially in terms of diatoms (274) and dinoflagellates (347). *Pseudo-nitzschia* species were frequent and abundant. In this study, conducted in 2010, we identified 6 *Pseudo-nitzschia* morphotypes, three of which were confirmed to be *P. pungens*, *P. subfraudulenta* and *P.* cf. *pseudodelicatissima,* other three were not confirmed (*P.* cf. *brasiliana*, *P. multistriata* and *P*. cf. *roundii*). The highest monthly mean abundances for *Pseudo-nitzschia* species (0.9×10^3 and 4.2×10^4 cells L^{-1}) was registered at the beginning of the rainy season (July) and during the cold-dry season (November), while the lowest values were recorded in the heavy rain season (mid-July-September) and in the driest month (May). According to the relative abundance, *Pseudo-nitzschia* showed higher contributions in July and in November, with values up to 20% of total phytoplankton. An specific relation with environmental parameters was not found.

Keywords: Acapulco Bay, Mexican Tropical Pacific, *Pseudo-nitzschia* spp., Bacillariophyta.

Introduction

Pseudo-nitzschia is a diatom genus that includes about 40 species; of these, 14 are producers of domoic acid which causes ASP (Lelong *et al*. 2012), generally when their concentration values are higher than 10^5 cells L^{-1} (Reguera 2002). In the Mexican Pacific region 15 species of *Pseudo-nitzschia* have been reported, of which 5 have been identified as toxic (labeled in bold): *P. americana, P. australis, P. brasiliana, P. delicatissima, P. dolorosa, P. fraudulenta, P. heimii, P. inflatula, P. lineola, P. micropora, P. multistriata, P. pseudodelicatissima, P. pungens, P. roundii and P subfraudulenta* (Hernández-Becerril 1998; Hernández-Becerril and Diaz-Almeida 2006; Gómez-Aguirre *et al*. 2004; Quijano-Scheggia *et al*. 2006). In the Tehuantepec Gulf, 10 *Pseudo-nitzschia* species have been recorded, including two recognized for the first time in the Mexican Pacific, *P. heimi* and *P. dolorosa* (Moreno-Gutiérrez 2008). In this study we provide information on *Pseudo-nitzschia* and their seasonal patterns in Acapulco Bay.

The Acapulco bay is located on the South of the Mexican Pacific coast (Fig. 1). It is a mesotrophic, small (7x10 km), deep (10-50 m) and semi-circular bay, with an "amphitheater" geomorphology. The bay receives several seasonal streams in the rainy season and consequently a large amount of waste and sewage from the densely populated surrounding hills. Acapulco Bay presents high phytoplankton richness (641 taxa; Meave-del Castillo *et al.* 2012).

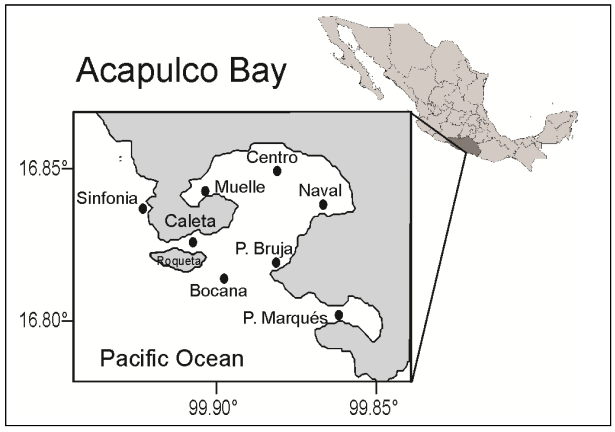


Figure 1. Study area and sampling locations.

According to monthly averaged atmospheric temperature and precipitation (Meave *et al.* 2012), three climatic periods are recognized in the Bay: warm-dry (March to May), rainy (June to September)

and cold-dry (October to February).

Methods

Samples were collected with a Van Dorn bottle in 8 sites (Fig. 1) at different depths (1, 3, 5, 10 m) and at the bottom (15-60m), bimonthly from October 2009 to January 2011. Samples were fixed with Lugol and Utermhöl chambers of 50 ml were used for cell counting (Ferrario *et al*. 1995). Diatom frustules were cleaned with hydrogen peroxide (30 %), samples were heated for 3 hours at 90°C (Reynolds 2014) and observed in TEM and SEM. Temperature, salinity and oxygen were measured with a multiparemetric sensor (YSI-556 MPS); nutrients were analyzed using standard procedures (Schwartz 1942, Solórzano 1969; Murphy and Riley 1962; Strickland and Parsons 1972). Chlorophyll *a* was estimated with a Spectrophotometric method (Parsons *et al*. 1984).

Results

Temperature ranged between 19.3 and 31°C, with the highest values (> 28° C) from June to October. Salinity ranged between 29.5 and 38.3, with the lowest values in the rainy season (July) and in December (cold-dry season). Dissolved oxygen ranged from 0.79 and 11.19 mg L^{-1}, with the highest values in October 2010, the lowest in the cold-dry season (February). Ortophosphate levels ranged from 0.02 to 13.6 µM, with the highest values in the rainy season. Nitrite + nitrate ranged between 0.05 and 28.2 µM, the highest values in the cold-dry season (November to February) and ammonium between 0.005 to 0.48 µM, with a slight peak in October (at the end of the rainy season). Silicates showed very low and constant values throughout the year (0.14-1.74 µM).
Bimonthly biomass ranged between 0.001 and 46.28 mg m^{-3}, with the highest values in March. Diatoms were abundant in Acapulco Bay mainly in the cold-dry season (October to January), with monthly mean densities between 5 x 10^4 and 9.1 x 10^5 cells L^{-1}. *Pseudo-nitzschia* spp. had the highest abundance in this season, with monthly mean densities ranging between 0.9 x 10^3 and 4.2 x 10^4 cells L^{-1}, corresponding to 24.5% (July 10^{th}) and 20% (November) of the total phytoplankton abundance (Fig. 2), and with a maximum monthly mean averange of 4.2 x 10^4 cells L^{-1} in November. Six *Pseudo-nitzschia* morphotypes were identified

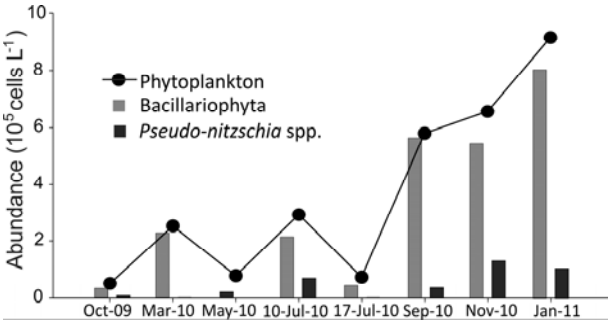
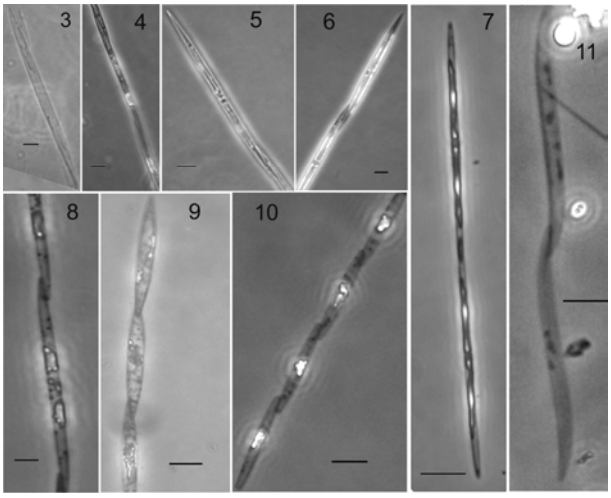
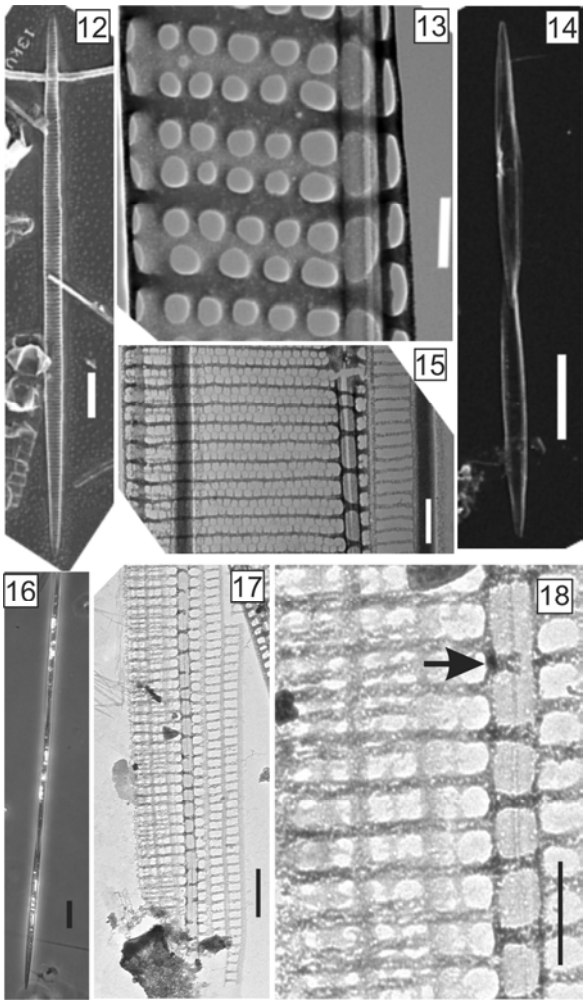


Figure 2. Monthly mean abundance of phytoplankton, diatoms and *Pseudo-nitzschia* spp. in the Acapulco Bay.



Figures 3-11. *Pseudo-nitzschia* species at the LM. Figs. 3-6, 8, 10-11) Cells in girdle view; Figs. 7, 9) Cells in valve view. Scale bars = 10 µm.

based on light microscope (LM) observations and morphometric characters (Table. 1), possibly corresponding to six species: *P. pungens* (Figs. 3-5), *P. subfraudulenta* (Fig. 6), a species of the *P. pseudodelicatissima* complex (Figs. 7-8), *P.* cf. *roundii* (Fig. 9), *P.* cf. *brasiliana* (Fig. 10) and *P. multistriata* (Fig. 11).
In the electron microscope, ultrastructural and morphometric characters (Table. 1) confirmed the identification of three species: *P. pungens* (Figs. 12-13), *P. subfraudulenta* (Figs. 14-15) and *P. cf. pseudodelicatissima* (Figs. 16-18). The *P. pseudodelicatissima* complex includes 8 taxa, several of which are cryptic or pseudocryptic. Our specimens match with the overlapping morphometric ranges of *P. cuspidata, P. pseudodelicatisima* and *P. manii* (Lundholm *et al.* 2012). In the present study the taxon was named as *P.* cf. *pseudodelicatissima,* because the species was already recorded for the



Figures 12-18. *Pseudo-nitzschia* specimens in LM (Fig. 16), SEM (Figs. 12-14) and TEM (Figs. 17-18). Figs. 12-13 *P. pungens*. Figs. 14-15 *P. subfraudulenta,* (arrow = central larger interspace). Figs. 16-18 *P.* cf. *pseudodelicatissima*. Scale bars: 13, 18 = 0.5 µm; 15, 17 = 1 µm; 12, 16 = 10 µm; 14= 20 µm.

region (Moreno-Gutiérrez 2008).
The monthly mean abundance of *Pseudo-nitzschia* morphotypes was variable throughout the year (Fig. 19). *P. pungen*s was abundant from July 2010 to January 2011 (Fig. 19). *P.* cf. *pseudodelicatisima* showed its maximum value in November 2010. *P. multistriata* was present in the rainy season with the highest abundance in July 2010. *P.* cf. *roundii* was only found in July 2010. *P.* cf. *brasiliana* was present in scarce densities along the dry season, but in July it had a peak of 5.7 x 10^4 cells L^{-1}. *P. subfraudulenta* was rare and present mainly from the end of the rainy season (September) throughout the cold dry season (Fig. 19). A relation with environmental parameters was not found.

Conclusions

Based on the recognition of different morphotypes, there are at least six species of *Pseudo-nitzschia* in Acapulco Bay. The identification of the most abundant species (*P. pungens* and *P.* cf. *pseudodelicatissima*), and also of the frequent species *P. subfraudulenta*, was confirmed by electron microscopy. As abundance values greater than 10^5 cells L^{-1}, can produce harmful effects when *Pseudo-nitzschia* species are toxic, it is important to monitor these species in Acapulco Bay when these diatoms are abundant, that is mostly during the rainy season, when orthophosphates show the highest values, and the cold-dry seasons, when nitrogen forms increase (Rojas-Herrera *et al.* 2012; Meave-del Castillo *et al.* 2012).
The occurrence of *P. multistriata* in Acapulco Bay represents a new record for the Mexican Pacific. This species is present in the North and South Atlantic, Mediterranean Sea, and in the Western Pacific. Confirmation is required for *P. roundii*, which was found in the Gulf of Tehuantepec, a region geographically close to Acapulco bay (Moreno-Gutiérrez 2008).

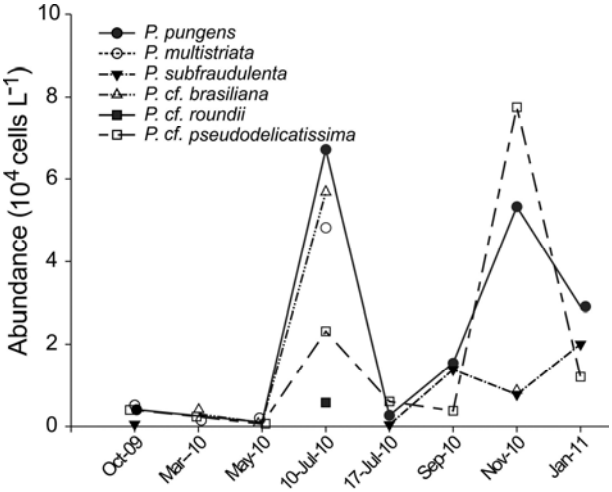


Figure 20. Monthly mean abundance of *Pseudo-nitzschia* spp. in Acapulco Bay.

Acknowledgements

We thank CONABIO for the financial support for project development HJ014. D.G-R. was funded by a scholarship from Posgrado en Ciencias Biológicas, UNAM.

Table 1. Morphometric characters of the species taken from: 1 Hernández-Becerril & Diaz-Almeyda 2006, 2 Lundholm *et al.* 2002, 3 Lundholmm *et al.* 2012, 4 Moschandreou & Nikolaidis 2010, Parsons *et al.* 2012, 5 Priisholm *et al.* 2002, 6 Quijano-Scheggia *et al.* 2008.

Pseudo-nitzschia **species**	**Valve outline**	**Girdle view outline**	**Apical axis**	**Trans-apical axis**	**Striae in 10µm**	**Fíbulae in 10µm**	**Pores in 1µm**	**Rows of pores**	**Central inter-space**	**Referen-ces**
P. brasiliana	rectangular	linear	12-65 **(26)***	1.8-3.4 **(3)***	20-28	20-27	7-10	2	Absent	2, 5, 7
P. multistriata	lanceolate	sigmoid	34-60 **(45)***	2.2-4 **(4)***	36-46	22-32	9-13	2-3	Absent	4, 7
P. pungens	linear-lanceolate	linear	74-160 **(85-115)***	2-4.5 **(3.2-6)***	9-16	9-16 **(12-15)***	3-5	2	Absent	4, 5, 7
P. roundii	asymmetric-lanceolate	asymmetric-lanceolate	53 **(43.5)***	6.3 **(4.5)***	31-32	16-18	4	1	Present	1
P. subfraudulenta	linear-lanceolate		65-106 **(55-65)***	5-7 **(3.5-4)***	23-26 **(28)***	14-17 **(16)***	2-6 **(5)***	2 **(2)***	Present	4
P. cf. *pseudodeli-catissima*	linear-lanceolate	linear	54-140 **(57-64)***	0.9-2.5 **(2-2.4)***	28-44 **(40)***	20-29 **(26)***	4-6 **(5)***	1-2 **(1-2)***	Present	3, 4, 5, 6

Bold and * = material from Acapulco Bay.

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Potentially toxic microalgae in a subtropical estuary and adjacent coast in Brazil with emphasis on the new record of *Nodularia spumigena* (32^0 S; 52^0 W)

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Abstract

The time series (February 1994 to May 2012) of potentially toxic microalgae data obtained in the scope of the Brazilian Long Term Ecological Research (BR-LTER) was analyzed, revealing the presence of at least 20 species in Patos Lagoon Estuary (PLE) and coastal adjacent Cassino Beach (CB) in southern Brazil. In the PLE the most abundant groups were freshwater cyanobacteria, followed by dinoflagellates and diatoms, while in CB, dinoflagellates, diatoms and raphidophytes were the main species. The analysis of the time series using decomposition tools aiming to extract the seasonal and interannual contribution for each variable, showed high interannual variability of the freshwater cyanobacteria without a clear seasonal pattern. The seasonal cycle of diatoms in PLE and CB presented a maximum in summer and minimum between August and October, while the dinoflagellates exhibited variable behavior throughout the year. We highlight the first record of the brackish non-native cyanobacterium *Nodularia spumigena* in CB in November 2011 and PLE in February 2012, following its novel blooms in aquaculture shrimp ponds in February 2011.

Keywords: Southern Brazil, Harmful species, time series, *Nodularia spumigena*

Introduction

The variability of phytoplankton composition and abundance in the Patos Lagoon Estuary (PLE) and Cassino Beach (CB), at the adjacent coastal area (southern Brazil), is driven by meteorological conditions: mainly by the rainfall amount in the basin and wind strength and direction (Abreu *et al*. 2010). In addition, chlorophyll *a* and primary production present a seasonal cycle, which is strongly related with the annual light variation (Abreu *et al.* 1995). In this area, the presence of potentially toxic microalgae including cyanobacteria, diatoms, dinoflagellates and raphidophytes (Table 1) represent a potential risk. At present, the extraction of natural bivalve banks for human consumption is small and the main aquaculture of shrimps and fishes is developed in ponds near the PLE. Nevertheless the production of microalgae toxins in aquaculture ponds may be a problem (Klein *et al*. 2011). The presence of potentially harmful species in the PLE and CB leads to the question of which is the most favourable period for their development and which species present the highest risk and should be treated with more attention.

Table 1. Potentially toxic microalgae species at the Patos Lagoon Estuary and Cassino Beach in southern Brazil.

Species	Reference
Dolichospermum sp. (*Anabaena*)	de Rosa *et al*. 2005
Microcystis aeruginosa	Yunes *et al*. 1998
Nodularia spumigena	New register
Trichodesmium erythraeum	Silva *et al*. 2008
Pseudo-nitzschia australis	Odebrecht *et al*. 2000
P. calliantha	Moreira 2004
P. multiseries	Odebrecht *et al*. 2000
P. pungens	Odebrecht *et al*. 2000
P. fraudulenta	Odebrecht *et al*. 2000
Alexandrium tamarense	Persich & Garcia 2003
Dinophysis acuminata	Odebrecht & Abreu 1995
D. caudata	Odebrecht & Abreu 1995
Gymnodinium catenatum	Persich & Garcia 2003
Lingulodinium polyedrum	New register
Noctiluca scintillans	Odebrecht *et al*. 1995
Prorocentrum minimum	Abreu *et al*. 1994
Protoceratium reticulatum	New register
Chatonella cf. *antiqua*	Odebrecht & Abreu 1995
Fibrocapsa japonica	Odebrecht & Abreu 1995
Heterosigma akashiwo	Persich et al. 1998

Material and Methods

The Patos Lagoon Estuary (PLE) and Cassino Beach (CB), in the adjacent coastal area, are subtropical environments in Southern Brazil (Fig. 1).

Fig. 1. Study area showing the Patos Lagoon Estuary (PLE) and Cassino Beach (CB), in Southern Brazil.

In the present work we analyzed the time series (February 1994 to May 2012) of data obtained through monthly sampling in the scope of the Brazilian Long Term Ecological Research (BR-LTER), comprising one point in Patos Lagoon Estuary (PLE) and a second one in the coastal adjacent area, Cassino Beach (CB). The time series analysis was performed using multiplicative decomposition methods of the "wq" package, part of the R v.2.14 software (R Core Team 2012). This method helps to separate the seasonal, interannual and random variability from the original series. Only data of genera including potentially toxic species were analyzed and the genera were grouped into general groups.

Results and Discussion

In the PLE the most abundant groups were freshwater cyanobacteria, followed by dinoflagellates and diatoms, while in CB, dinoflagellates, diatoms and raphidophytes were the most expressive (Figure 2). The diatom *Pseudo-nitzschia* spp. was one of the most frequent harmful species complex in both environments (14.5-27.7%). In PLE, the fresh water cyanobacterium *Dolichospermum* spp.

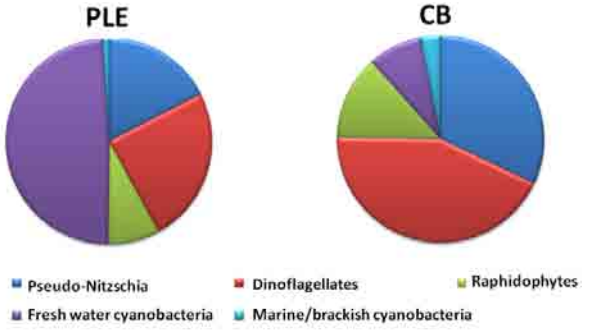

Fig. 2. Relative contribution of taxonomic groups at the Patos Lagoon Estuary and Cassino Beach.

Table 2. Occurrence frequency (%) of potentially toxic genera and species at the Patos Lagoon Estuary and Cassino Beach.

Organism	CB (%)	PLE (%)
Pseudo-nitzschia spp.	27.7	14.5
Alexandrium spp.	4.1	4.5
Dinophysis. acuminata	11.4	2.7
Dinophysis caudata	7.3	0.5
Gymnodinium catenatum	1.4	0.5
Lingulodinium polyedrum.	0.5	0
Prorocentrum minimum	6.8	9.5
Protoceratium reticulatum	0.5	0
Noctiluca scintillans	23.6	5.9
Dolichospermum spp.	2.3	15.9
Microcystis spp.	2.7	6.4
Nodularia spumigena	0.5	0.5
Trichodesmium spp.	2.3	0.5
Raphidophytes	12.3	6.8

(15.9%), *Microcystis* spp. (6.4%) and the dinoflagellate *Prorocentrum minimum* (9.5%) were also important, while in CB the dinoflagellates *Noctiluca scintillans* (23.6%), *Dinophysis. acuminata* (11.4%), *D. caudata* (7.3%) and raphidophytes (12.3%) were the most frequent. In the PLE, raphidophytes (6.8%) and *N. scintillans* (5.9%) and *Dinophysis* spp. (3.2%) were less frequent (Table 2).

We highlight the first record of the brackish non-native cyanobacterium *Nodularia spumigena* in CB in November 2011 and PLE in February 2012 (Fig. 3a), following novel blooms in aquaculture shrimp ponds in February 2011 (Fig. 3b). The blooms in the aquaculture ponds led to shrimp mortality and affected their growth and reproduction (Klein *et al.* 2013). The mechanism of the recent introduction of *N. spumigena* is unknown and could include

Fig. 3. *Nodularia spumigena* microphotographs from (a) natural environment (Patos Lagoon Estuary) in February 2012 and (b) from Aquaculture station bloom in February 2011.

natural ways via migrating birds from Uruguay (Pérez *et al*. 1999) or further south in Brazil (Werner and Rosa 1992) or ship ballast water. The decomposition analysis of the time series (Fig. 4) showed high interannual variability for all groups, reaching more than four times the contribution of the seasonal component. Freshwater cyanobacteria didn't present a clear seasonal pattern. The seasonal cycle of diatoms (*Pseudo-nitzschia* spp.) in PLE and CB presented a maximum in austral summer and minimum between August and October, while dinoflagellates exhibited variable behavior throughout the year and lowest concentration in late summer (Feb- March) in CB and in spring/early summer in PLE. Raphidophytes presented a similar annual cycle in both environments, with maximum in early summer (Nov-Dec) and minimum in late summer (Feb-March). Marine/brackish cyanobacteria decomposition analysis showed inconsistencies, possibly due to the main marine species, *Trichodesmium erythraeum*, which occurs episodically, and the brackish *Nodularia spumigena*, which has been registered only recently in this environment. The highly variable behavior of organisms observed over time is probably related to the high hydrodynamics of the region.

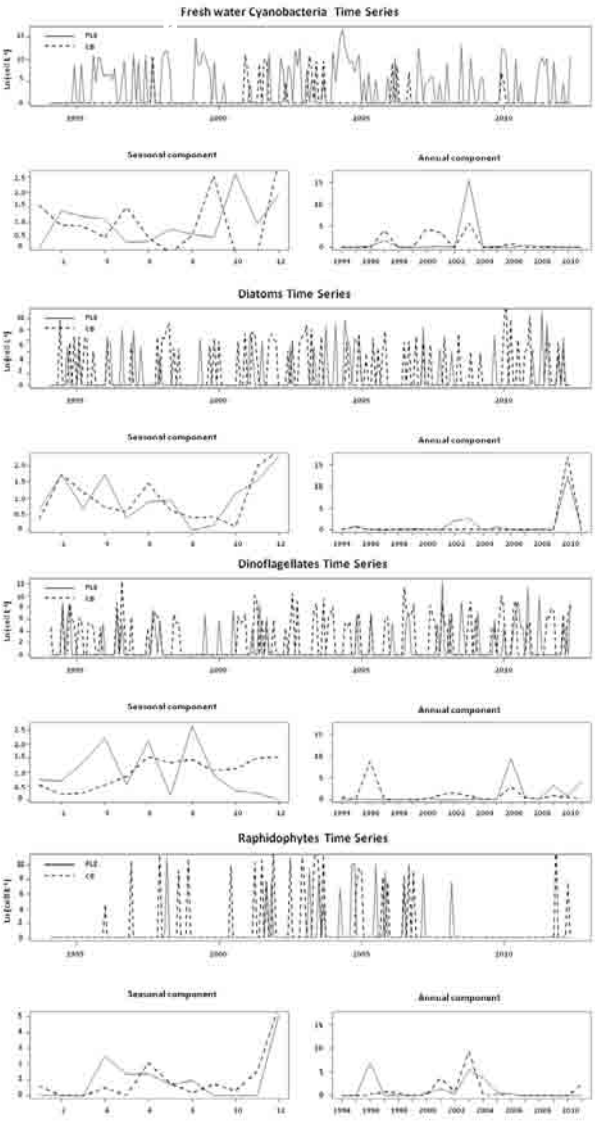

Fig. 4. Time series plots and resulting decomposition analysis (seasonal and annual components) for fresh water cyanobacteria, Diatoms, Dinoflagellates and Raphidophytes in PLE and CB.

Conclusions

The presence of several harmful species represents a potential problem in the Patos Lagoon Estuary and Cassino Beach environments. Time series analysis revealed that harmful species are present all the year around and *Noctiluca scintillans*, *Dinophysis* spp., *Pseudo-nitzschia* spp. and *Dolichospermum* spp. are the most frequent ones. The recent introduction of noxious *Nodularia spumigena* has led to a problem in shrimp ponds and may have negative impacts in future.

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Red tide outbreaks in Alexandria (Egypt) waters: Invasive harmful species

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Abstract

A distinct increase has been observed along the Egyptian Mediterranean coast of Alexandria in the frequency of red tide blooms, their intensity, magnitude, geographical extent and number of causative species. The area is subject to significant amounts of untreated wastewaters from land-based sources, rendering the water highly eutrophic. During 1997-2012, 71 red tides were reported between late spring and early autumn, of which 51 were studied. The bloom duration, and its species composition and environmental conditions varied with 4% of the bloom peaks collapsing within a couple of days, 70-85% maintained for three to five days, and 11-26% lasting for more than a week. The blooms (24 species) appeared mono-specifically or in combination. Cases of massive invertebrate and fish mortality were triggered at 6 periods, the worst in Aug 2004 and July- Aug 2005, as caused by *Alexandrium ostenfeldii*, *Gymnodinium catenatum*, *Heterocapsa circularisquama*, *Karenia mikimotoi*, *Prorocentrum minimum*, and the raphidophycean *Chattonella antiqua*. Toxic species introductions are increasing, and 8 putative invasive species out-compete native species and seriously reduce diversity in the area; the potential role of ballast water is discussed

Keywords: harmful algal blooms, eutrophication, invasive harmful species, Alexandria

Introduction

"Invasive species" can be defined by multiple ways (Lodge *et al*. 2006) including any introduced species reported that rapidly colonize and spread outside its native range (e.g., Occhipinti-Ambrogi and Galil 2004), and threatens abundance and native biological diversity (Lamoreux *et al*., 2006), the ecological stability of ecosystems, economic activities dependent on these ecosystems and/or human health (EPA 2001). However, others recommend that the term invasive should have a stricter biological definition without any inference to an organism's impact (Falk-Petersen *et al*. 2006), since ecological impacts might be modified by anthropogenic stressors (Byers 2002). Here we examine trends of red tide blooms in Alexandria waters**,** selective ecological forces that allow their outbreaks, and the success of introduced species to establish blooms. The interaction with other phytoplankton species, and trophic levels and losses of biodiversity were investigated. The studies suggest that Alexandria exports invasive species to other Mediterranean countries. Some remedial processes are discussed.

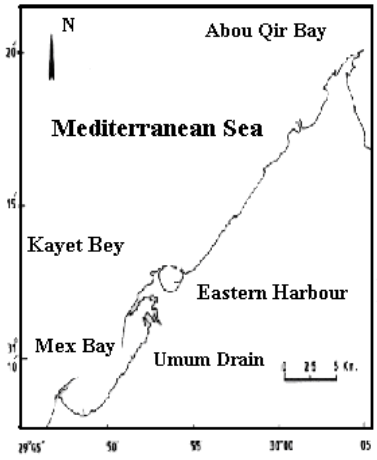

Fig. 1. The study area.

Study area.

The investigated area (Fig. 1) extends 50 Km along the Egyptian coast of Alexandria, includes estuaries, harbors and semi-enclosed basins. It is subjected monthly to significant amount of untreated wastewaters ($183x10^6 m^3$) from different land-based sources, rendering the water highly eutrophic. The hot spots of pollution are: Mex Bay (19.4 km^2) a marine

transitional estuarine system W Alexandria (6.5x 10^6 m^3 d^{-1} of wastewaters); the Eastern Harbour in the central part of the city (2.53 km^2); Dekhila Harbour (12.5 km^2) a part of Mex Bay; and Abu Qir Bay east of Alexandria, a shallow semicircular basin (50 km long) subjected to daily land run-off and fresh water from the Nile Rosetta mouth. The data presented here (Labib 2000, 2009; Labib and Mikhail 2007; Mikhail 1997, 2001, 2003a,b, 2007; Mikhail and Halim 2008; Mikhail *et al*. 2005, 2008) is based on the monitoring programme of phytoplankton and environmental conditions carried out in Egyptian

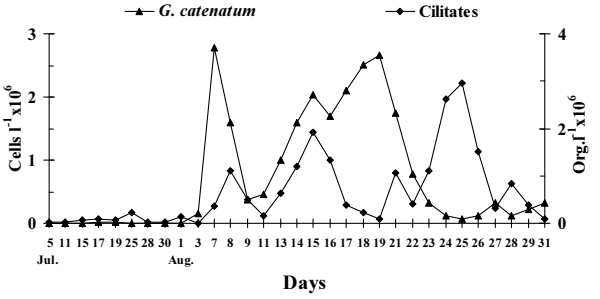

Fig. 2. Ciliate assemblages and *G. catenatum* during July-August 2004

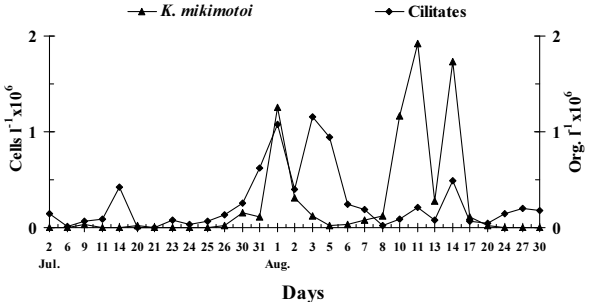

Fig. 3. Ciliate assemblages and *K. mikimotoi* during July-August 2005

Mediterranean waters of Alexandria during the years 1997 to 2010.

Results and Discussion

Eutrophication. The land-runoff discharges rich in organic substances and anthropogenic nutrients are the main cause of man-made eutrophication in Alexandria waters. Nutrient input west of Alexandria (t $month^{-1}$) is estimated as 177-238 NH_4, 22-51 NO_3, 21-26 PO_4, and 526-1141 SiO_4. Eutrophication may be responsible for the blooming of invasive phytoplankton species (Pertola *et al*. 2005), causes shifts in the species composition of ecosystems, while changes in nutrient ratios might explain the variability in phytoplankton species diversity in Alexandria. Increased numbers of *Melosira*, *Rhizosolenia*, *Euglena* and *Eutreptiella* spp., and the decrease in diatom: flagellate ratio might serve as an indicator.

Recent trends of Red Tide blooms in Alexandria waters. HAB species have been regarded as invasive. The recent trends include: i) Only a few scattered areas were previously affected by HABs, but over the last 2 decades, virtually every coastal area has major blooms; ii) Regular events of increased recurrent red tide outbreaks from late spring-summer and fall. The blooms in 2001 offer a good example as 16 algal species (9 diatoms, 5 dinoflagellates, 1 euglenophycean and 1 raphidophycean species) were responsible for the massive blooms at 8 intermittent periods from mid-May to late Oct; iii) The replacement of the native *Alexandrium minutum* (last massive bloom in Oct 1994, Labib and Halim, 1995) by other harmless/harmful bloom formers, mostly of newly introduced dinoflagellates, and raphidophytes; iv) The increasing frequency of blooms of *Prorocentrum triestinum* and *P. minimum*. The last bloom of the first on 16 Aug 2007 contributed 38.76x10^6 cells l^{-1} at 19.8-29.5°C Salinity 22.5-37.5, while the second at 7.3x10^6 – 9.15x10^6 cells l^{-1} in early Sept 2007. v) The apparent increase of several migratory/invasive phytoplankton species/ blooms; vi) The massive blooms of unidentified microflagellates in late winter-early spring, late summer-early autumn; vii) The multispecies blooms became common since 2009, and monospecific blooms disappeared; viii) The diatom, *S.* cf *costatum*, a major constituent of recent blooms, contributed massive blooms, the last in June 2012 (222.5x10^6 cells l^{-1}), ix) Episodic blooms of indigenous diatom species not previously known to form massive occurrences such as *Asterionellopsis glacialis* (1.2x10^6 cells l^{-1}, Feb. 2000), *Rhizosolenia setigera* (13.39x10^6 cells l^{-1}, May 2006), *Lithodesmium undulatum* (2.7x10^6 cells l^{-1}, June 2007), *Cyclotella nana* (10.8x10^6 cells l^{-1}, July 2007), *Leptocylindrus mimimum*, *Rhizosolenia delicatula*, *Nitzschia longissima*, *Chaetoceros socialis* (10.2x10^6 cells l^{-1}, Sep. 2007) and *Rhizosolenia fragilissima* (14.19x10^6 cells l^{-1}, July 2009); x) The steady increase in the numbers of indigenous dinoflagellates previously sparsely reported: *Dinophysis acuminata*, *Akashiwo sanguinea, Gonyaulax spinifera, Prorocentrum sigmoides*, *Ceratium furca, Cochlodinium polykrikoides*

and *C. Catenatum*; xi) The recent occurrence of the putative alien species, *Alexandrium catenella* and *Heterosigma* sp.; xii) The progressive occurrence and geographical distribution of the invasive species *C. antiqua*. Its peaks in late Aug-early Sept 2006 (23.13×10^6 cells l^{-1}, Chl *a* 88.5 μg l^{-1}) were 45 fold- that of the first bloom in late Sept 1998 and 15 fold values in May 2001. This species had a wide spatial distribution (20 km) with a maximum cell density at $13.1x10^6$ cells l^{-1}, and maintained at 27−30°C and 34.6-37.5 psu; xiii) The blooms in July-Aug 2004-2005 were unique as including six toxic species, *Gymnodinium catenatum* and *K. mikimotoi* were the major constituents (Table 1). The sharp changes in nitrogen−silicon ratio led to a large increase in the number of blooms of invasive phytoplankton species that do not require silica for their growth such as *G. catenatum*.

Invasive species as harmful Algae in Alexandria waters. Studies of invasive phytoplankton species in Alexandria waters revealed increasing frequency, intensity, geographic spread, and duration. The following species were recorded the last 15 years: *Alexandrium catenella*, *A. ostenfeldii*, *Chattonella antiqua*, *Gymnodinium catenatum*, *Karenia mikimotoi*, *Heterocapsa circularisquama Heterosigma* sp., *Pseudo-nitzschia australis*, *P. pungens*.

Reverse migration. Between 1960 and 1994, *Alexandrium minutum* was the most common red tide in Alexandria. For unknown reasons, after its last massive bloom in Oct 1994 (Labib and Halim 1995) the species almost disappeared. By the mid 1980s, blooms of *A. minutum* increament in other parts of the Mediterranean Sea have been related to human transport, natural current patterns, nutrient loading and aquaculture (Lilly *et al*. 2002; Vila *et al*. 2001).

Success of invasive species. The factors enabling alien species to establish permanent populations cannot easily be identified. Physical (e.g., high winter temperatures, thermo-haline stratification) and chemical factors (excess of nutrients, and shifts in nutrients ratios) may be involved. The lag times between introduction (e.g., *Gymmnodinium catenatum*) and its maximum occurrence can span years. Reasons for the apparent increase of invasive species have been proposed to include increased awareness, cultural eutrophication, ballast water and ship fouling vectoring, and reclaiming of coastal areas (Pertola *et al*. 2006).

Ecological problems. The ecological impacts are complex and dependent on the interaction between the invader and the native community. We have observed: i) Incidents of fish and invertebrate mortality in the Eastern Harbour during summer 1998-1999; July-Aug 2004, 2005 with blooms of *G. catenatum* and *K. mikimotoi*, during late Aug/ early Sept2006, and 2010 with *C. antiqua*, in Sept 2007 with *P. minimum*, and in 2010 with *A. ostenfeldii;* ii) The occurrence of C. *antiqua* in late May 1999 affected the species diversity; *S.* cf *costatum* and *Asterionella glacialis* dominated prior to the bloom, and *Thalassionema nitzschioides* after its collapse. By contrast, diatom numbers were severely reduced on the bloom peak day, while dinoflagellates grew well; iii) The bloom of *G. mikimotoi* in May-June 1999 was preceded by a massive bloom of *S.* cf *costatum*. High counts of *Cyclotella nana* and unidentified microflagellates accompanied the bloom period. *Protoperidinium depressum* and *Scrippsiella trochoidea* dominated after the bloom. The data indicate a strong impact on phytoplankton diversity; iv) *Thalasssiosira* spp. dominated the community before the triggering of *C. antiqua*, in combination with *P. minimum* in Sept 2001. Diatoms were poorly diversified, while dinoflagellates were numerous within the bloom. The excess of nutrients after the bloom collapse permitted a well-diversified community (32 species). v) The blooms of *G. catenatum* and *C. antiqua* in Aug 2006 and *A. ostenfeldii* and *K. mikimotoi* in early July and Aug 2007 caused severe decrease in other accompanying species. However, *P.pungens* succeeded within the bloom periods; vi) The inter-specific relation between microalgae blooms, co-occurring heterotrophic and mixotrophic dinoflagellates and ciliates was examined in 2004-2005, when the six species *A. ostenfeldii*, *G. catenatum*, *H. circularisquama*, *K. mikimotoi*, *P. minimum*, and *C. antiqua* were the causative species. Co-occurring ciliates (33 genera, 56 species), heterotrophic and mixotrophic dinoflagellates (20 species) were affected. However, the bloom developed without regulation due to ciliate grazing. Ciliate and mesozooplankton abundances were minimal during major peaks of *G. catenatum*, and *K. mikimotoi* blooms, indicating avoidance and/or low grazing pressure (Figure 2 & 3). Versatility of resistance to grazers and predators is one of the features that determine the success of invasion by an organism. Killing the nutrient-competing phytoplankton species enable these species to utilize limiting resources such as nitrogen and phosphorus, accelerating the processes for alien phytoplankton species to become invasive

ones. Understanding the links between human and natural disturbance and massive development of newly, induced species will help prevent marine bio-invasions.

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Table 1. Cell densities of the major species and accompanied temperature and salinity.

Date		Causative species	Temp.(˚C)	Cells/L x10^6	Salinity
2004					
July	30	*P. minimum*	28.5	0.23	33
August	6	*P. minimum*	28.4	0.88	36.5
	7	*G. catenatum*	28.2	2.78	35.5
	19	*G. catenatum*	28.8	2.67	34
		C. antiqua		1.74	
	21	*G. catenatum*	27.8	1.74	34
2005					
July	2	*P. minimum*	26.5	0.56	37
	25	*A. ostenfeldii*	27.4	0.52	35.8
	30	*G. catenatum*	27.3	1.56	37
	31	*A. ostenfeldii*	28	0.52	37.5
August	11	*K. mikimotoi*	28.5	1.92	34.2
	14	*K. mikimotoi*	28.2	1.73	36.5
		C. antiqua		0.48	
	24	*Heterocapsa sp.*	27.5	0.40	36

Accumulation and transformation of paralytic shellfish toxin by the pen shell *Atrina pectinata*

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Abstract

The bivalve molluscs *Atrina pectinata*, *Crassostrea gigas, Mytilus galloprovincialis* and *Perna viridis* were naturally contaminated with paralytic shellfish toxins (PST) during an *Alexandrium catenella* bloom. The accumulation of toxins in those shellfish was analyzed by HPLC with fluorescence detection. Toxin content in *A. pectinata* was relatively low compared to that in other bivalve species. Anatomical distribution of PST was investigated in *A. pectinata* and the toxin concentration in adductor muscle was very low compared to other organs. Only adductor muscle from this species is used as food; therefore the removal of other organs is an efficient way for assuring seafood safety. In contrast, kidney had a high concentration of PST; GTX5 was the predominant toxin, accounting for 69.4-82.7 mol%. Analysis of 28 strains of *A. catenella* isolated from the same waters showed that GTX5 was predominant only in one strain. These results suggest that the predominant GTX5 in *A. pectinata* kidney might be transformed from another toxin.

Keywords: Paralytic shellfish toxin, pen shell, *A. pectinata*, *Alexandrium catenella*, GTX5

Introduction

The benthic infaunal pen shell *Atrina pectinata* is a commercially important species in Asian Pacific areas including western Japan, but little attention has been paid to its possible accumulation of phycotoxins. In Ariake Bay (Kyushu Island), the main production area of this bivalve, over 10,000 t of pen shell were harvested per year before the 1970s. But the production rapidly decreased and remained about 100 t after 1999 (Kawahara and Ito 2003). The situation led to inceased interest in culturing the species by methods developed in Japan (Maeno *et al.* 2009). Culturing *A. pectinata* in coastal areas poses a risk of PST accumulation from the causative dinoflagellates. In terms of food safety, it is therefore important to investigate the accumulation of PST in *A. pectinata*, because the cultures are suspended below the water surface. In this study, *A. pectinata* cultures were suspended below the seasurface during a bloom of *A. catenella*, and the anatomical distribution of PST analyzed by HPLC with fluorescence detection.

Materials and Methods

Bivalves infested with PST
A. pectinata, M. galloprovincialis, *P. viridis* and *C. gigas* were suspended below the water surface (0.5-1.5m depth) at the pier of Shin-Nagsaki fishing port from May 13 to 25 in 2011. After suspension for 13 days, four specimens of each bivalve were collected for PST analysis by HPLC. Cell density of *A. catenella* in the surface water at the pier was determined by microscopic cell counts during the experiment.

Sample preparation for HPLC analysis
Live specimens of *A. pectinata* were shelled and separated into 7 parts (adductor muscle, mantle, gonad, gills, digestive gland, kidney and foot) and each part extracted with 0.1 M acetic acid and centrifuged at 10,000 g for 10 min. Then the supernatant was treated with a Sep-pak C18 cartridge and filtered with Ultrafree C3 (0.2 μm) for HPLC analysis. Three other species of bivalves were shelled and the whole soft tissue analyzed according to the same procedure.

Analysis of toxin profile of *A. catenella*
Twenty-eight strains of *A. catenella* were isolated from seawater collected on 30 May at the pier of Shin-Nagsaki fishing port. These isolates were cultured in f/2 medium and the toxin profiles of the cultured cells were analyzed by HPLC with fluorescence detection.

HPLC analysis: PST content in the bivalves and in the causative dinoflagellate *A. catenella* were Table 1 Toxicity scores of bivalves placed below the water surface at the pier of Shin-Nagasaki fishing port.

Species	Toxicity scores(MU/g)
A. pectinata	1.2 ± 0.4
C. gigas	2.9 ± 0.6
M. galloprovincialis	28.8 ± 4.5
P. viridis	7.3 ± 1.5

determined by HPLC-FD (Oshima 1995) using PST standards, specifically GTX1, GTX2, GTX3, GTX4, decarbamoyl GTX2 (dcGTX2), decarbamoyl GTX3 (dcGTX3), C1, C2 STX and neoSTX. Bivalves toxicity, expressed in mouse units (MU) per gram, was calculated from the toxin content from HPLC analysis and the specifc toxicity of each PST determined by Oshima (1995). The specific toxicity is the estimated value of (MU)/µmol for each toxin and 1 MU is the amount of toxin required to kill a 20g ddY strain male mouse in 15 min after intraperitoneal injection. 4 MU/g, equivalent to 80µg/100g STX, is the regulation limit for bivalves in Japan.

Results and discussion

Cell density of *A. catenella* during the suspension experiment ranged from 508 to 30,000 cells/l (Fig 1). The four bivalve species were naturally contaminated with PST for 13 days during the bloom and their toxicity calculated from results of HPLC analysis (Table 1). PST levels in the pen shell *A. pectinata* (1.2 ± 0.4 MU/g) were lower than those of the oyster (2.9 ± 0.6 MU/g) and the mussels *M. galloprovincialis* (28.8 ± 4.5 MU/g) and *P. viridis* (7.3 ± 1.5 MU/g). Our investigation showed that the oyster was less toxic than mussels in the natural environment. This is in agreement with previous works regarding differences in toxin

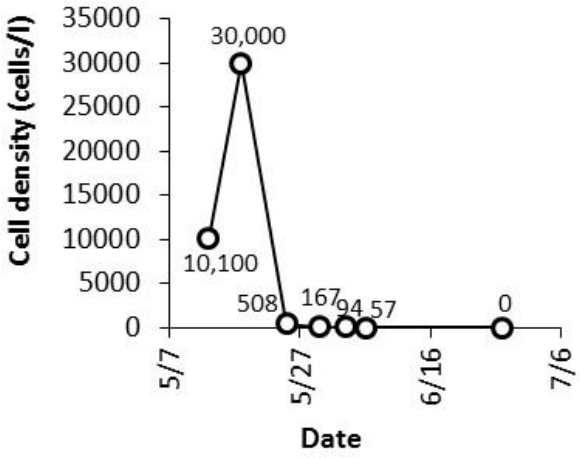

Fig. 1 Cell density of *A. catenella* in the surface waters during the experiment.

accumulation among bivalve species. (Shumway 1990; Bricelj and Shumway 1998). Toxicity of scallop *Patinopecten yessoensis* and *Chlamys nipponensis,* which are also important commercial species in Japan, was always high compared to those of mussel *M. edilus* (Oshima *et al*. 1982). These results suggest that the pen shell *A. Pectinata,* among important bivalve species of Japan, would be a low risk species for PSP toxins.
Anatomical distribution of PST in *A. pectinata* was also analyzed and the toxin concentration found in the adductor muscle was very low compared to that in other organs (Fig. 2). In *A. pectinata* only the adductor muscle is used as food, therefore the removal of other organs is an efficient way to assure seafood safety. Kidneys had a high concentration of PST, with GTX5 as the predominant toxin accounting for 69.4-82.7 mol% (Fig 3). Toxin profiles of *A. catenella* strains isolated from the same waters

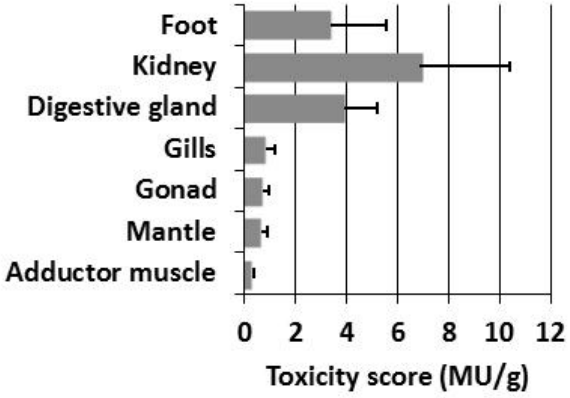

Fig. 2 Anatomical distribution of PST in *A. pectinata.*

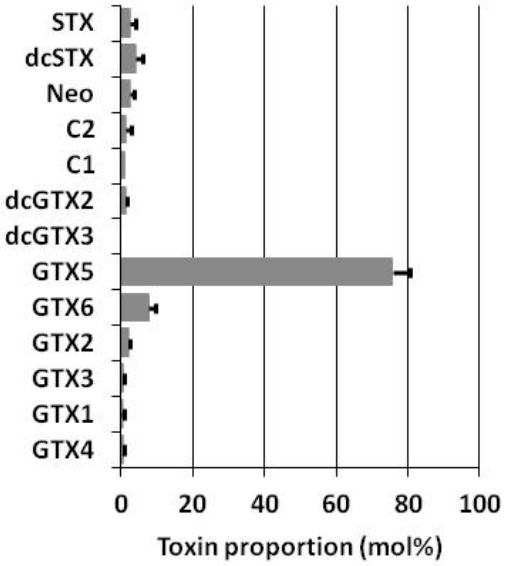

Fig. 3 PST toxin profile in the kidney of *A. pectinata*.

as the suspended bivalves were also analyzed, but GTX5 was predominant only in one out of the 28 isolated strains (Fig. 4). Although the possibility of selective accumulation of GTX5 should be considered, this difference suggests that the GTX5 might be transformed from another toxin in *A. pectinata*.

Acknowledgement

We express our gratitude to Dr Oshima of Kitasato University, Dr Noguchi of Tokyo Healthcare University, Dr Ryuichi Watnabe of NRIFS and the Food Safety and Consumer Affairs Bureau in MAFF for providing the PSP toxin standards. This study was partially supported by the Regulatory research projects for food safety, animal health and plant protection funded by the Food Safety and Consumer Affairs Bureau in MAFF.

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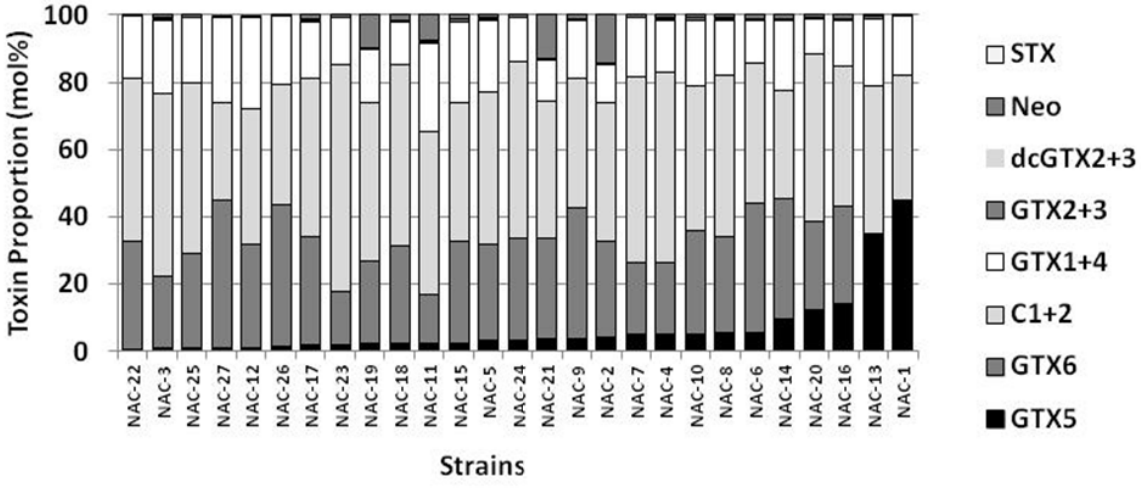

Fig. 4: PST toxin profiles of *A. catenella isolated from the pier of Shin-Nagasaki fishing port on 30 May*.

Recent HAB events in Ha Long Bay (Vietnam): Increase in frequency, harmful effects associated with increased eutrophication

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Abstract

In the two years, 2011-2012, at least five red tides were formed by various species, including *Ceratium furca* (July 2011). *Phaeocystis globosa* (November 2011), *Noctiluca scintillans* (April 2012), *Gonyaulax polygramma* (May 2012) and *Chattonella* sp. (August 2012). Of those, the second and third species were for the first time recorded to form red tides in the north of Vietnam while the last two were for the first record in bloom proportion in Vietnamese waters. This is a dramatic increase in frequency, intensity and variety of HAB events, considering that during the last 10 years, only one red tide was observed (2008) after the first event in history of the western coast of the Tonkin Gulf was officially recorded in June 2002. These blooms lead to serious losses in aquaculture. The total loss is estimated to be about 3-5 million USD, including 3,000-5,000 tons of bivalves (*Meretrix lyrata*) and 20 tons of various species of caged fish such as cobia (*Rachycentron canadum)*, snapper (*Lutjanus argentimaculatus* and *L. erythropterus*), grouper (*Ephinephelus* spp.) and seabass (*Lates calcarifer*). The increase in red tide frequency corresponded with the increase nutrient level and abundance of phytoplankton. Concentrations of N, P, which are measured in the form of NO_2^-, NO_3, NH_4^+ and PO_4^{3-}, have sharply increased by 2-4 fold while phytoplankton abundance has increased 20-300 times during 2005-2012.

Keywords: red tide, *Ceratium furca, Noctiluca scintillans, Phaeocystis globosa,* Ha Long Bay.

Introduction

Ha Long Bay is a world heritage site located in the north of Vietnam. This is not only a well-known tourist destination but also an important aquaculture area. Like many other parts of Vietnam, in the past, this Bay was free from HAB threats and the term "red tide" was unfamiliar to local people as well as researchers. However, the situation changed since 2002, when the first red tide in western Tonkin Gulf (Nguyen *et al*. 2005) was observed in Cat Ba Island. Since then, the phenomenon of water discoloration, here in referred as "red tide", and eutrophication became a highly concerned issue and frequently included in various studying and monitoring programs such as Thuoc (2002), Khanh (2011). In this paper we present the results of a two-year (2011-2012) monitoring on red tides and eutrophication in the southern part of Ha Long Bay with emphasis on red tide occurrence. Phytoplankton abundance and nutrient level are examined and compared with previous data to clarify the situation of pollution and harmful algae in the area.

Material and Methods

Four sampling sites representing marine water (Cat Ba Harbour and Ben Beo), brackish water (Phu Long) and mesohaline water (Vinh Quang) were monitored regularly during February 2011-September 2012 (Fig. 1).

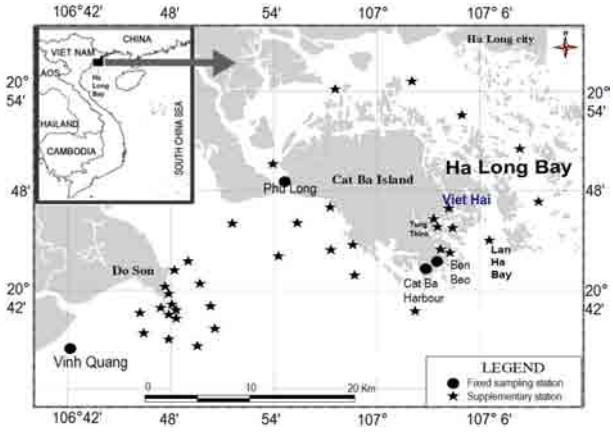

Fig. 1: Map showing locations of sampling sites.

Sampling frequency was every two weeks for Phu Long and Vinh Quang sites and weekly for Cat Ba Harbour and Ben Beo. When a red tide occurred, additional sites were sampled to explore the bloom magnitude. Qualitative samples were collected by phytoplankton net (30 cm diameter; meshsize 20µm). Quantitative samples were taken by filling up plastic bottle of 2-litter with surface water. All samples were fixed with acidified Lugol's iodine solution (3ml/L). Cell density was counted using a Sedwick-Rafter chamber under a Nikon E600 fluorescent microscope. Nutrients (NO_2^-, NO_3^-, NH_4^+, Si-SiO_3^{2}, PO_4^{3-}) were analyzed by spectrophotometry method (APHA *et al*. 2005) using a DRELL/2010 HACH spectrophotometer.

Results and discussion

Red tide by *Ceratium furca*

During Feb. 2011 - Sep. 2012, at least five red tides were observed. The first red tide was by *C. furca*. The affected area was the eastern side of Cat Ba Island (Ben Beo station and surrounding area), where a large number of fish cages is located. The bloom lasted for two weeks (20th July -4th August 2011) and coincided with the death of 11 tons of fish (cobia, snapper, grouper and seabass).

Earlier, *C. furca* has been recorded to form red tides in the same area during June-August in 2002 (Nguyen *et al*. 2005) and 2008 (Thuoc, personal communication). Their cell densities were detected to reach over million cells/l and large-scale water discolor was observed. This implies that blooms of this species usually occur in the middle of summer (June-August). The intervals between bloom events by this species (2002, 2008 and 2011) also suggest an increase in frequency of red tides in recent years. *C. furca* is well known as a species which is capable of forming red tide worldwide. Its bloom has been observed in many regions in the world such as Japan (Okachi 2003), China (Yan and Dai 2000), South Korea (Lee and Huh, 1983), Kuwait (Glibert *et al.* 2001), Mexico (Guerramartinez and Laravilla, 1996) and South Africa (Anderson *et al.* 2001). According to Gentien *et al.* (2003), aquatic animals have been killed by red tides of this species and the cause is suggested to be oxygen deficiency and gill clogging.

Red tide by *Phaeocystis globosa*

During 15th-18th November 2011, a red tide by *P. globosa* was detected in the neritic waters of the south-western Cat Ba Island.

It is uncertain about the original of the bloom, but it is clear that China and Vietnam share the water masses of the Gulf and thus effort to study and mitigate HAB should base on collaborative efforts.

P. globosa was found to be in spherical colonies with diameters up to 1cm, which formed a thick layer of mucus settling on the seabed (fig. 3). A large proportion of aquatic animals such as hard clam and *Lingula* sp. were killed. The bloom also led to the loss of about 2000 tons of hard clams (*Meretrix meretrix)* cultured in the area. Previously, bloom of *P. globosa* had been recorded in the southern waters of Vietnam (Binh Thuan Province),

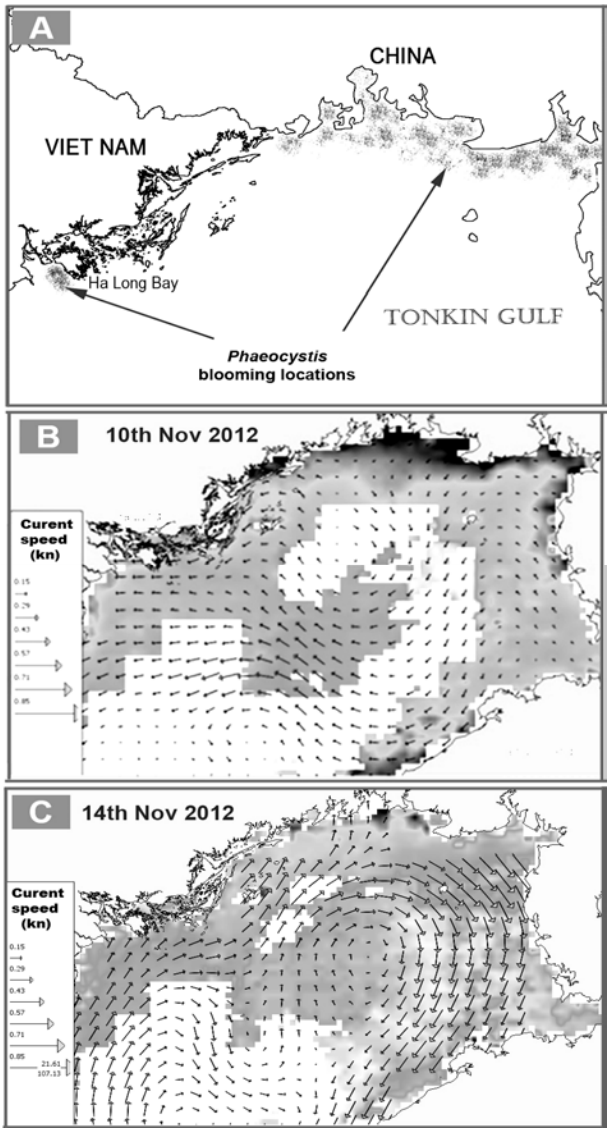

Fig. 2: Maps showing locations of *P. globosa* bloom in Chinese and Vietnamese coastal waters during November 2011 (A) and the surface currents of the same period on 10th November (B) and 14th November (C).

where they formed foam near shore and devastated an area of 40km^2 (Hai *et al*. 2010; Lam 2010). However, this is the first record of this species in the northern waters of Vietnam.

P. globosa is well known as a "brown tide" species which usually forms massive blooms in cold or temperate, neritic waters (Schoemanna *et al.* 2005). Its colonies can produce acrylate in the mucus layers which can poison marine animals as the colonies separate (Noordkamp *et al*. 1998).

It is uncertain about the scale of the bloom due to the lack of large-scale monitoring data. However, according to Jiang and Chen (of Guangxi Academy of Sciences, personal communication), during the same period, a large-scale bloom of *P. globsa* was also observed along the coastal waters of China (Fig. 2A). This suggests that the bloom might have been a larger scale event, covering a large portion of Tonkin Gulf. Oceanic currents (Fig. 2B, 2C), which could reach up to 0.4 knot (Fig. 2C), showed that the Gulf was under a strong and unstable circulation during that period. Under such conditions, water masses can be quickly transported between Chinese and Vietnamese waters and could possibly travel between the blooming sites in matter of 2-3 weeks, considering the distance between blooming sites in Ha Long Bay and the one in China coast is about 130 miles (clockwise) to 300 miles (unclockwise).

Red tide by *Noctiluca scintillans*

During 28th March to 16th Apr. 2012 a large-scale red tide was formed by red *N. scintillans*. The bloom extended in the whole western part of Ha Long Bay. In protected areas, it formed a dense surface layer with a thickness of 4-5cm and the highest density reached up to 4.6 million cells l^{-1}. In open waters, patches of red tide of up to 1000 m were found as far as 15km offshore. In the tidal zones, red tide patches settled down and formed "reddish pools" during the neap tides. Consequently, a large of proportion of wild and cultured benthic animals was killed. Around 3000 tons of hard clams, which values approximately 3 million USD, cultured around Cat Ba Island was killed.

Red *N. scintillans* has frequently been recorded in the north of Vietnam but this is the first red tide ever recorded. In southern waters of Vietnam, where water is warmer, blooms of green *N. scintillans* have been recorded by Lam (2010) in Van Phong Bay. The difference in distribution patterns of green and red *N. scintillans* in Vietnam waters provides a good example of the geographic boundaries of the two strains within one species. This distribution pattern

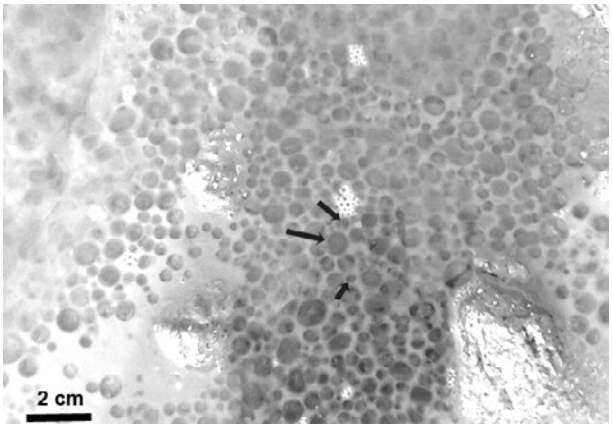

Fig. 3: *Phaeocystis* colonies formed mucus layer settling on seabed in Cat Ba Island during November 2012.

is in agreement with previous authors (Harrison *et al.* 2011) who claimed that the tolerance temperature range for green strain of *N. scintilans* (25°C - 30°C) is higher than that of the red strain (10°C - 25°C).

Other red tides

Besides the three red tides described above, two localized red tides of smaller scales were also observed in the protected areas. During 19th-23rd May 2012, *Gonyaulax polygramma* bloomed in close water masses of the eastern side of Cat Ba Island. However this bloom resulted in no fish kills, despite extremely high density (27 million cells·l^{-1}) was observed. Despite this species being very common, this is the first record of red tide of *G. polygramma* in Vietnamese waters.

During late July and early August 2012, at the same sites where blooms of *G. polygramma* took place, a brown tide of *Chattonella* sp. was observed with maximum densities of 2.7 million cell·l^{-1}. The bloom led to a loss of about 3 tons of cobia (*Rachycentron canadum*) and grouper (*Epinephenus* spp.) cultured in the area. This is the first record of *Chattonella* species in Vietnamese waters.

The increase of phytoplankton abundance

Data obtained by this study shows that there has been a dramatic increase in abundance of phytoplankton recently. Mean density of phytoplankton in 2011-2012 (670,000 ± 2,500,000 cells/l, n=130, maximal 27 million cells/l) is about 22-156 times higher than that of the years 2004-2005 (16,540 cells/l ± 30,000 cells/l, n=24, according to Thuoc (unpublished data).

Red tides connection with eutrophication

The occurrence of 5 red tides in a duration of less than two years implicates a dramatic increase in frequency of HAB events. The increase corresponds

well with the increase in level of the two major nutrients, N and P. Measurements of these nutrients in form of NO_2^-, NO_3, NH_4^+ and PO_4^{3-} (Fig. 4) show strong increase by 2-4 folds during 2005-2012, with exception to NO_2^-, which is not stable in the water and can be easily shifted to NO_3.

The nutrient increase in the Bay, as showed in this study, is in agreement with previous authors such as Tac (2006), Tung (2006) and Thanh (2008). Tonkin Gulf is a semi-closed gulf surrounded by populated provinces of China and Vietnam. As the economy of these area continue to develop, the pressure on environment of the Gulf will continue to rise in the coming year. Ha Long Bay is also heavily affected by the local pollution as a result of aquaculture development. The amount of fish cage in the area, according to Khanh (2006), has been far beyond the carrying capacity of the area. If there no immediate effective countermeasure is taken, nutrients will continue to rise and frequency and intensity of red tides in the area will likely to increase.

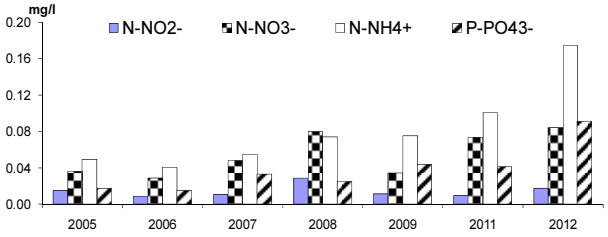

Fig. 4: Dissolved nutrients in Cat Ba seawater during 2005-2012. (Source: 2005-2009: Khanh, (2011); 2011-2012: this study).

Conclusions

We present evidence showing an increase in frequency and intensity of HAB events in Ha Long Bay. This increase corresponds well with the increase in nutrient levels in the region. Red tide have caused severe threats for aquaculture and the ecosystem in Ha Long Bay. The problem of red tide is more severe than was previously thought

Acknowledgements

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HAB monitoring, modeling, and prediction

Phytoplankton variability modulation by the hydrodynamic regime in Alfacs Bay (NW Mediterranean). A combined experimental and modelling study

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Abstract

Understanding the spatio-temporal variability of phytoplankton in aquaculture areas is necessary for the appropriate management of natural resources and the prevention of toxic outbreaks. With this objective, we combined synoptic cruises, time series of physical parameters, and modeling, to study the ecosystem of Alfacs Bay, an important shellfish and fish production area commonly affected by toxic outbreaks.
Synoptic cruises performed during relevant harmful species proliferations, such as a *Karlodinium* spp. outbreak in 2007, showed the existence of a preferential phytoplankton accumulation area in the inner NE side of the Bay. We explored the role of nutrient supply (which takes place mainly through the irrigation channels discharging into the northern coast) and the hydrodynamic regime in explaining the observed phytoplankton distribution patterns. Based on a 3D hydrodynamic model combined with a particle-tracking module, we suggest that the phytoplankton confinement in that area could be fostered by the estuarine circulation dynamics taking place in the bay.

Keywords: Alfacs bay, NW Mediterranean, phytoplankton dynamics, estuarine circulation

Introduction

Alfacs Bay is a shallow microtidal estuary located in the Ebre Delta (NW Mediterranean), which receives freshwater inputs coming mainly from the runoff of rice field irrigation channels discharging from its northern coast, and from groundwater seepage (e.g. Camp and Delgado 1987). Its high productivity (in contrast to the adjacent oligotrophic Mediterranean waters) has allowed the development of valuable aquaculture activities. Unfortunately, recurrent harmful phytoplankton outbreaks (caused mainly by *Alexandrium minutum*, *Dinophysis* spp., *Pseudo-nitzschia* spp. and *Karlodinium* spp.) threaten this industry (Fernández-Tejedor *et al.* 2008). In this context, the appropriate management of natural resources and the prevention of toxic outbreaks require a good understanding of the phytoplankton dynamics in the bay and in particular of harmful algal blooms.
With this objective in mind, we investigated the spatio-temporal variability of the phytoplankton biomass distribution in relation to the major physico-chemical forcings: nutrients, general circulation, light availability, and degree of stratification (Llebot *et al.* 2010 and references therein). The motivation for this study derived from our observation of maximum abundances of the ichthyotoxic *Karlodinium* spp. on the NE side of the Bay and near and below the pycnocline during an outbreak in June-July 2007. In subsequent field cruises and by combining both *in situ* sampling and modeling tools, we explored the underlying mechanisms which could lead to this preferential phytoplankton accumulation area in the bay.

Materials and methods

Synoptic cruises (1- to 2-day duration) were performed to obtain *in situ* data to characterize the variability of both the phytoplankton biomass (chlorophyll, cell numbers) and the physico-chemical parameters (water temperature and salinity from CTD casts and organic and inorganic nutrients from water samples) at basin scale. The cruises were conducted between April 2007 and July 2011, at different times of the

year covering the three main periods regarding the magnitude of freshwater inputs (open, semi-open, and closed irrigation channels). Some cruises coincided with the occurrence of harmful outbreaks. Modeling experiments were performed to test hypotheses regarding the observed basin-scale phytoplankton distributions and variability. A semi-implicit 3D hydrodynamic model of water circulation (Smith 2006) previously implemented in Alfacs Bay (see detailed description in Llebot *et al.* accepted) was coupled to a Lagrangian particle-tracking model (Ross and Sharples 2004, Ross *et al.* in prep). The models had been previously validated using continuous time series data of water temperature, salinity, water velocities, and fluorescence obtained with instruments moored at a central station near the mussel rafts, combined with concurrent meteorological information. The simulations were performed with 2 clouds of 4000 passive tracers. One cloud was released in a vertically homogeneous distribution near the mouth of the bay and the second cloud in the bay's interior. Simulations were performed for relevant periods for which we had cruise data, in order to compare the observed chlorophyll distributions with the modeled tracer concentrations.

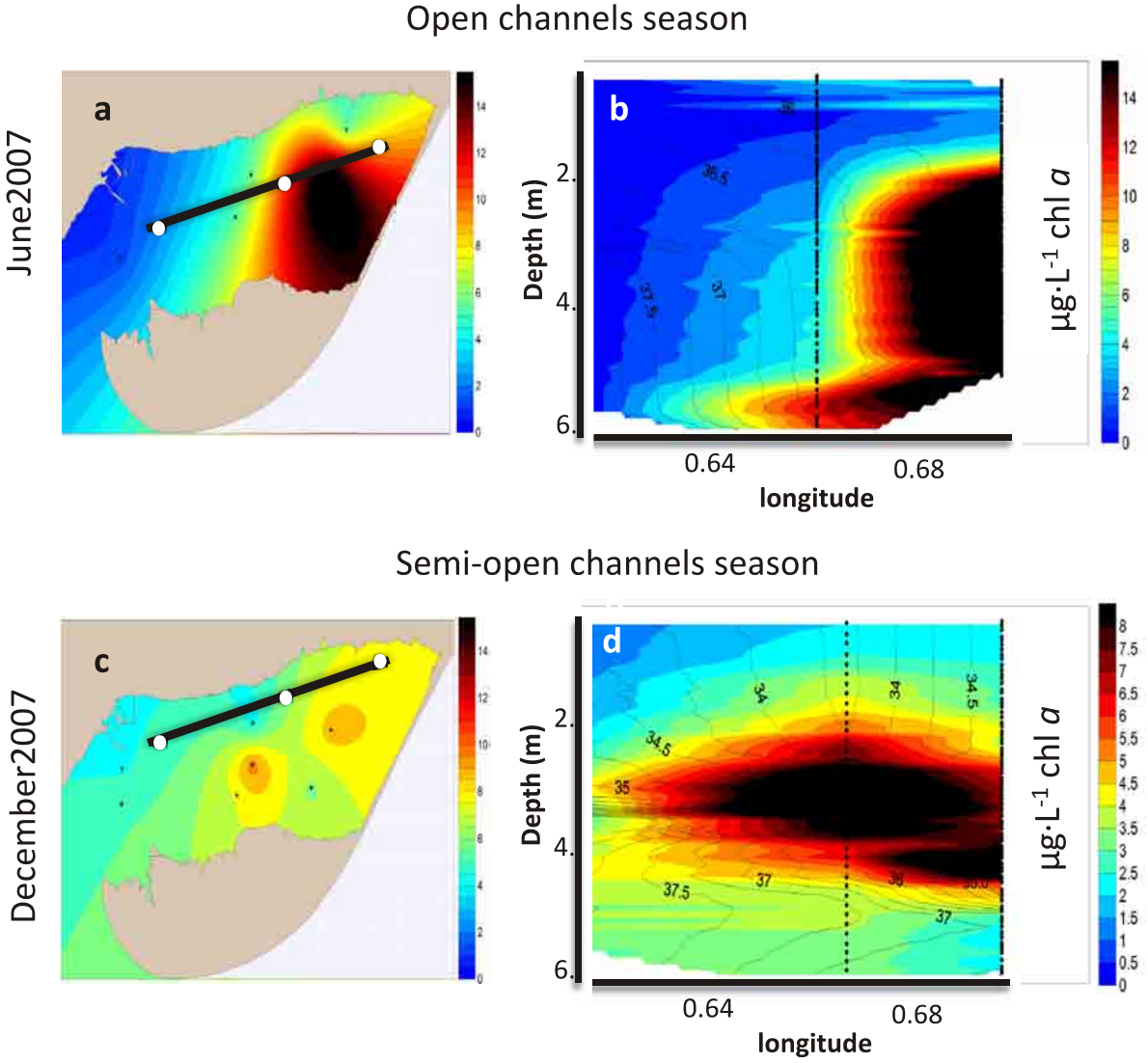

Fig. 1: (a, c) Distribution of depth averaged profiles of chlorophyll a in Alfacs Bay in representative cruises. Dots indicate the sampling points. (b, d) Vertical distribution of chlorophyll a along the longitudinal transect of the bay (black bar in a and c). In these graphs chlorophyll is plotted in colour contours with overlays of salinity contours.

Results and Discussion

At a basin scale, horizontal salinity and density gradients were often observed in Alfacs Bay as reported in previous studies (not shown, but see e.g. Camp and Delgado 1987, Llebot *et al*. 2014).
Less dense, brackish waters (salinity <35) were typically found in the northern interior of the bay, while dense, saltier (salinity range 36-37.8) marine waters would be observed to enter from the Mediterranean Sea. Along the year, the water density varied as a result of temperature and salinity fluctuations. The highest density values (26.5 − 27.7) were measured during the coldest winter months (December to March) with a reduced freshwater inflow from the channels (semi-open and closed channels periods). In turn, the lowest densities (22-26) were typically recorded during the warmer summer months of June and July (open channels period).
The phytoplankton distributions in the bay exhibited a rather heterogeneous pattern. For instance, in June-July 2007 the highest chlorophyll concentrations occurred towards the NE, in the interior part of the bay (Fig. 1a). In the vertical, chlorophyll maxima were mainly found just above the pycnocline or near the bottom (Figs. 1b). *Karlodinium* spp. reached maximum concentrations of ca. $1.5x10^{-6}$ cell·L^{-1}, while chlorophyll increased up to 30 µg· L^{-1}. During a subsequent survey in December 2007 (Figs. 1c and 1d), a similar horizontal pattern was observed. In that case, the only toxicogenic organism, *Dinophysis* spp., was present below the warning threshold. In general, the highest phytoplankton biomass was associated with brackish rather than with marine waters.
Measurements of nutrient concentrations confirmed that the main supply enters through the irrigation channels (Camp and Delgado 1987) with comparatively low levels during the closed channel period (Llebot *et al*. 2010). The highest concentrations were typically observed near the N (Sant Carles port) and NE interior of the bay. However, areas with high nutrient levels did not always coincide with high chlorophyll concentrations, suggesting that other factors may modulate the phytoplankton patches in the bay.
In particular, we tested whether the hydrodynamic flow regime could facilitate the existence of a retention area favoring biomass accumulation. The particle tracking simulations showed how the estuarine circulation and tracer distributions responded to changes in buoyancy, wind and tidal forcing. The estuarine circulation was particularly active when the water column was stratified and wind mixing was weak (Fig. 2a). In this situation, the circulation drives the bottom particles towards the bay's interior, while flushing the particles in the surface layer out into the open Mediterranean. When wind-induced mixing is strong, the density stratification and associated estuarine circulation break down (Fig. 2b) and particles accumulate in the bay's interior (Fig. 2c). These model results are in good agreement with observed distributions of chlorophyll (Fig. 1), supporting the hypothesis that the inner bay retention area is largely controlled by physical forcing. The degree of tracer retention is lower when the channels are open (e.g. July 2007, figs. 1a and 1b) compared to the closed and semi-open periods (e.g. December 2007, figs. 1c and 1d). When the freshwater inflow from irrigation channels is reduced (semi-open or closed channel periods) and/or wind-induced mixing is high, water column stratification decreases resulting in a weakening of the estuarine circulation. The residence time (defined as the time for which at least 50% of the particles remain in the bay) in such situations is of the order of 3-4 weeks which is a period long enough to facilitate bloom development. This often coincides with *Alexandrium minutum* and *Dinophysis* spp. outbreaks (Fernández *et al*., 2008). In addition, nutrient levels can also be relatively high in this period. A direct link between nutrients and circulation cannot be established at this stage, as concentrations result from the balance between sources (including recycling) and consumption by the organisms. When the estuarine circulation is well developed (often coinciding with the open channel period), nutrient levels are high but the residence time can drop to less than 1 week.
Some other studies to characterize in more detail the seasonal and small-scale variability of phytoplankton patchiness, including harmful species, are in progress (Artigas et al. in press). Future research is aimed to accurately calculate the residence times under different circulation scenarios. In addition to an improved understanding of Alfacs Bay circulation, results here highlight the importance of physical/ hydrographic factors in determining HABs.

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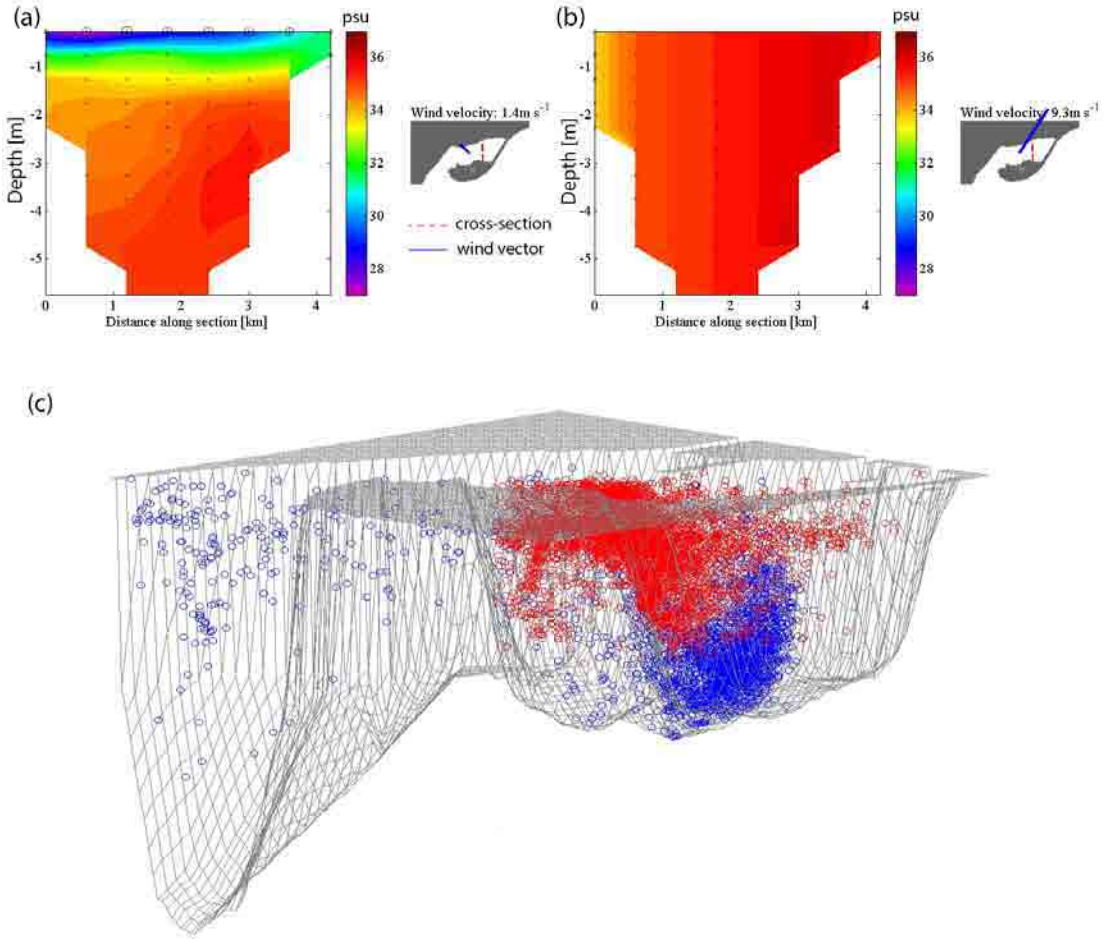

Fig. 2: Simulation of a semi-open channel scenario showing (a) the vertical salinity stratification during low wind intensities (the crosses and circles with dots inside them, represent the flow into and out of the bay respectively), (b) higher wind leading to weakened salinity stratification and a breakdown of the flow, (c) a snapshot of the coupled 3D hydrodynamic-particle tracking model including the bottom topography showing the retention of two clouds of particles (blue and red circles) in the NE interior.

Comparative studies on phytoplankton dynamics and bio-optics for HAB monitoring in the Ebro Delta, NW Mediterranean

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Abstract

Alfacs and Fangar bays are semi-enclosed embayments in the Ebro Delta system, where sustainable shellfish aquaculture requires harmful algal bloom (HAB) surveillance at the appropriate spatio-temporal scale. We therefore deployed a hyperspectral light-field observational system in Alfacs Bay, in late spring through summer over two successive years. The vertical distribution and bloom dynamics of two HAB-genera, the toxigenic dinoflagellate *Karlodinium* spp. and the diatom *Pseudo-nitzschia* spp. were assessed by weekly laboratory analyses of phytoplankton and ancillary parameters in a comparative approach in both bays. Bio-optical measurements provided continuous time-series proxy data for changes in phytoplankton biomass adjacent to aquaculture sites. Small scale dynamics of *Karlodinium* spp. and *Pseudo-nitzschia* spp. displayed a patchiness that could be related to temperature and salinity patterns. While we do not consider these parameters as direct forcing functions, they are related to other relevant parameters, such as stratification and nutrient regime, and thus may act as proxy indicators for bloom initiation. Continued studies on a species level are necessary to fully assess bloom dynamics for this region, but future integration of both bio-optical and phytoplankton patterns at the sub-mesoscale will support regional oceanographic models for HAB forecasting and enhance surveillance.

Keywords: coastal embayments, bloom patchiness, remote sensing reflectance, spatio-temporal coverage.

Introduction

The two semi-enclosed embayments Alfacs and Fangar bays in the Ebro Delta system are the major aquaculture sites in Catalonia (NW Mediterranean). Bivalve shellfish, predominantly the Mediterranean mussel *Mytilus galloprovincialis*, are grown on fixed rafts in suspension culture. Due to the presence of harmful microalgal taxa and accumulation of their phycotoxins, both bays are subject to occasional harvesting closures. Semi-confined environments, such as the Ebro Delta bays are characterized by an increased retention time of water and associated plankton, and are more influenced by point source input (e.g., of nutrients or fresh-water) than open coastal systems exposed to long-shore advection. These conditions can lead to increased stratification and therefore increased vertical patchiness of phytoplankton proliferations. Consequently, the surveillance of HABs must adequately address bloom patchiness at an appropriate spatio-temporal resolution (Cembella *et al.*, 2005). Bio-optical tools have gained prominence in synoptic and long-term assessment of HABs (see Babin *et al.*, 2008), especially for documentation of the presence, dimensions and movement of blooms. Ocean colour analysis is widely applied to track chlorophyll *a* (Chl *a*) as an algal biomass proxy (Stumpf *et al.*, 2003), although this approach provides poor taxonomic resolution and it is not often possible to distinguish harmful from benign blooms.

In the Ebro Delta, the ichthyotoxic dinoflagellate *Karlodinium*, represented by co-occurring species, *K. armiger* and *K. veneficum* (formerly referred to as *Gyrodinium corsicum*, Garcés *et al.*, 2006), has caused marine faunal mortalities (Fernández-Tejedor *et al.*, 2010). An increase in cell abundance of *Pseudo-nitzschia* spp. has also been observed in the region over the past 20 years (Fernández-Tejedor *et al.*, 2010), including detection several species known to produce the neurotoxin domoic acid (DA). Even though the presence of DA has not led to shellfish harvesting closures, the increase in abundances

poses a future threat to the regional shellfish industry. The comparison of HAB dynamics in two semi-enclosed embayments is an approach to increase knowledge on bloom dynamics. The ultimate objective of the current study is to address the spatio-temporal patchiness of HABs and to determine which factors regulate the population dynamics of diverse HAB taxa in these multi-faceted marine environments. We applied a hyperspectral light-field observational system for continuous monitoring of phytoplankton in Alfacs Bay and assessed the dynamics of target genera in the two bays to address this objective.

Field sites and sampling strategy

Field work was conducted in the Ebro Delta bays from May to July in 2010 and 2011. Remote sensing reflectance (R_{rs}) was continuously retrieved throughout both study seasons with a radiometric sensing system installed on an aquaculture raft in Alfacs Bay (40.6200830 °N, 0.6581670 °E) (Fig. 1). R_{rs} was calculated via $R_{rs\,(\lambda)} = [L_{sfc} - 0.024 \times L_{sky}] / E_d$, where a percentage of the sky radiance L_{sky} was subtracted by the factor 0.024 from surface upwelling radiance L_{sfc} to obtain water leaving radiance (L_w). The L_w was then divided by the solar plane irradiance E_d. Spectra influenced by surface objects and strong sun glint were deleted and remaining sun and sky reflections were corrected following J. Busch *et al.* (2013).

The analysis of biological and physical parameters was conducted weekly at a vertical resolution of 0.5 m in Alfacs and Fangar Bay (40.7787667 °N, 0.7492333 °E). This included CTD (conductivity, temperature, depth) casts as well as water samples for laboratory analyses of nutrients with an auto-sampler, and the phytoplankton community by means of inverted microscopy. The extracted Chl *a* (Chl a_{extr}) content of surface samples was determined fluorometrically as reference to bio-optical measurements.

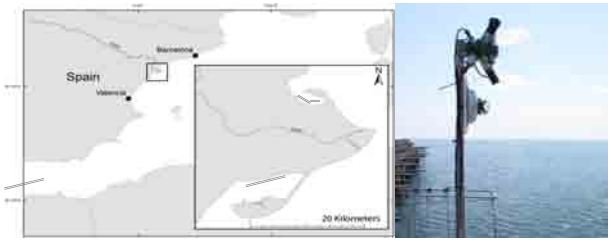

Fig. 1: Left: Study site in the Ebro Delta on the NW Mediterranean coast within the two semi-enclosed embayments Alfacs (northern) and Fangar (southern); Right: sensor system setup with radiometers for the near real-time assessment of anomalies in chlorophyll *a* as algal biomass proxy and a camera system to check surface and sky conditions.

Continuous observation of phytoplankton biomass

Retrieval of Chl *a* as phytoplankton biomass proxy by means of R_{rs} measurements was approached by an algorithm employing the ratio [R_{rs} (555 nm)$^{-1}$ × R_{rs} (490 nm)] (Fig. 2), despite interference of coloured dissolved organic matter (CDOM) at these spectral ranges. Algorithms targeting the absorption peak of Chl *a* in the red region (~674 nm), to avoid masking of the signal by CDOM (Dall'Olmo and Gitelson, 2005), as well as those targeting sun-induced natural Chl *a* fluorescence from the R_{rs} signal, failed in explaining variations of reference Chl a_{extr}, (J. Busch *et al*, unpublished). The radiometer system was operated with a 15 min sampling interval and therefore recorded variations in phytoplankton biomass with a high temporal resolution in a fully automated mode. Values complied well with fluorometrically derived Chl a_{extr} from weekly sample extracts that were used for algorithm development. In addition, an external dataset from a nearby station, derived from a local weekly routine monitoring programme for food safety conducted by the IRTA, was used as validation dataset for the applied algorithm, and could be aligned to the bio-optical data (Fig. 2).

Highest Chl *a* concentrations were measured at the end of the 2010 study season. Even though increased cell abundances of *Karlodinium* spp. were also identified by microscopic counts, this information could not be isolated by data from the bio-optical system. eter system measured spectra at hyperspectral resolution (3 nm steps). Such measurements allow retrieval of detailed spectral data on phytoplankton pigment absorption and based on this information more details on algal composition. As an example, high cell abundances of the toxic dinoflagellate *Karenia brevis* in the Gulf of Mexico have been identified from bio-optical data by an inversion of R_{rs} to phytoplankton absorption spectra over the spectral range (Craig *et al.*, 2006). The identification was based on distinct absorption characteristics of *K. brevis* due to the presence of the rare pigment gyroxanthin-diester. This technique may also be applicable for high biomass and “mono-generic” blooms of *Karlodinium* spp. in the Ebro Delta bays, if these dinoflagellates also dominantly contain this rare pigment.

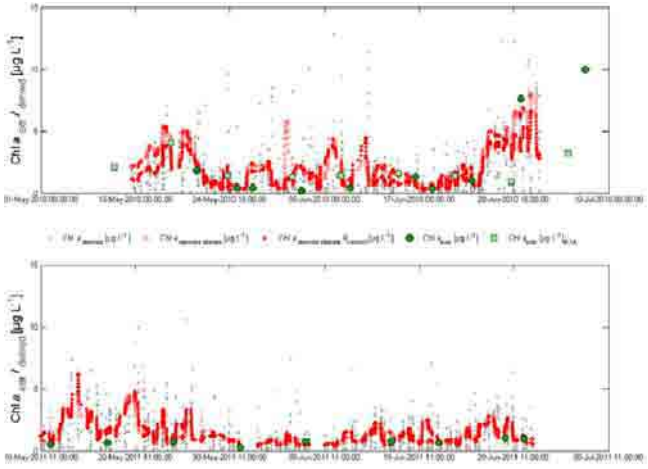

Fig. 2: Phytoplankton biomass proxy retrieved by reflectance measurements in Alfacs Bay over the study time in 2010 (upper) and 2011 (lower panel). Remaining influences of sun and sky reflections (grey), filtered by means of a moving average (red) complied well with laboratory measurements (Chl a_{extr}, dark green circles). Corresponding Chl a_{extr} values at a nearby station were not included in algorithm development, but also complied well with the overall pattern (light green squares), only available for 2010.

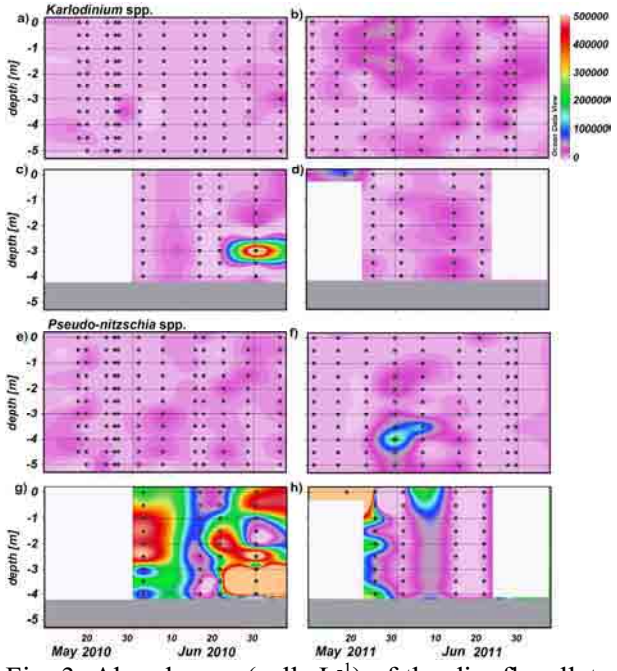

Fig. 3: Abundances (cells L^{-1}) of the dinoflagellate *Karlodinium* spp. and the diatom *Pseudo-nitzschia* spp. in Alfacs (a, b, e, f) and Fangar (c, d, g, h) bays in 0.5 m vertical steps (black dots represent water samples) for the years 2010 and 2011.

Phytoplankton bloom dynamics

In Alfacs and Fangar bays, the stratification regime is mainly formed by freshwater inflow from rice-field irrigation channels. Due to irrigation patterns, an increased seasonal intensity was expected during the study time. A freshwater layer in the surface meter was apparent in both bays over the whole study period in 2011, whereas in 2010 it was restricted to Alfacs Bay in the beginning of May. While an assemblage of the two target genera due to this layer was not visible, an irregular vertical patchiness pattern was clearly shown (Fig. 3). In 2010, the highest cell counts of *Karlodinium* spp. were from samples from around 3 m depth in both bays, with highest abundance on 1 July 2010 in Fangar Bay. A general correspondence of high cell abundances within a window of temperature (20 – 27 °C), as apparent with data from January to August 2010 (Busch *et al.*, 2012), was basically confirmed with this dataset. Cell abundances of *Pseudo-nitzschia* spp. were patchy at various depths, but an exceptional bloom occurred in Fangar Bay in May 2011 (> 7×10^6 cells L^{-1}). This event could only be captured from surface sampling during harsh wind and wave conditions that presumably initiated the decline phase of the bloom. *Pseudo-nitzschia* cell abundances exceeded the alert level (2×10^5 cells L^{-1}) from 9 to 30 May, by which time concentrations still remained rather high 5.4×10^4 cells L^{-1}). A redundancy analysis was conducted to examine if the target genera *Pseudo-nitzschia* and *Karlodinium* indeed respond similarly to the increased freshwater inflow in both bays. For both bays, variances of *Pseudo-nitzschia* spp. occurrences could be best explained by salinity (freshwater), whereas variances of *Karlodinium* spp. were associated more closely with temperature (Fig. 4). These factors are not considered key elements that trigger blooms of these genera, but are related to other factors, such as stratification or nutrient input, and may aid in outlining proxies for HAB dynamics.

All results presented here are shown on a genus level, as the discrimination of species within these genera by light microscopy is problematic. Further information on the species level is required to address questions regarding which biotic and abiotic factors control population dynamics, because even populations within a single species may express different adaptive strategies for growth and survival that determine bloom dynamics. Preliminary results on the species composition of *Pseudo-nitzschia* by means of 454 DNA sequencing show the presence

of a local species mixture with potentially toxic and non-toxic taxa (J. Busch *et al.*, unpublished). This complies well with prior findings for the area (Andree *et al.*, 2011; Quijano-Scheggia *et al.*, 2008). Along the Catalan shellfish production areas, DA has been detected in shellfish, but was below quantification level in the Ebro Delta Bays most of the time (Giménez *et al.*, 2012). Whereas both *K. armiger* and *K. venificum* are prominently associated with HAB events in the Ebro Delta, it is therefore not clear which *Pseudo-nitzschia* species are responsible for the production of DA in this area. This is one of the questions that will be addressed in the next step of this study.

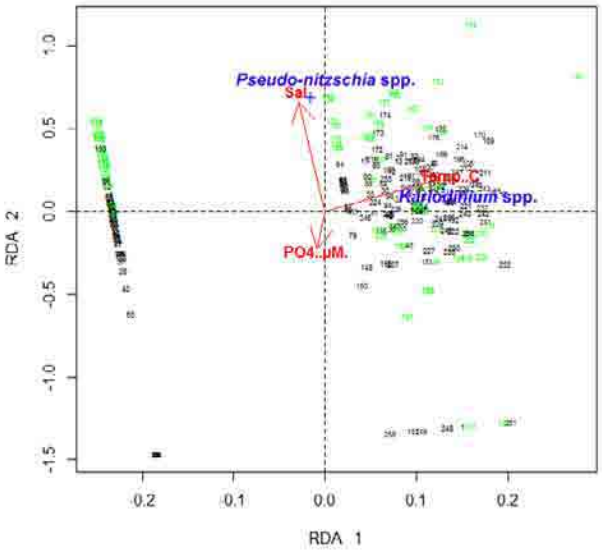

Fig. 4: Redundancy analysis of *Pseudo-nitzschia* and *Karlodinium* spp. in Alfacs Bay (black) and Fangar Bay (green), for the variables salinity, temperature and inorganic phosphorus.

Conclusions and future perspectives

An automated long term retrieval of phytoplankton biomass by a radiometric sensor system was successfully applied in the Ebro Delta to record the presence of algal blooms. Inclusion of such a system into an environmental observatory provides a synoptic view of phytoplankton blooms over the appropriate spatio-temporal scales for aquaculture installations. Strategic location for an observatory is critical for areas identified as bloom incubators. For example, NE Alfacs Bay may function as bloom incubator for *Karlodinium* spp. due to increased retention time of water (Berdalet *et al.*, this volume). Taxon specificity is as mandatory for HAB surveillance as a large spatio-temporal coverage, but the former cannot be determined by this regional bio-optical approach. The inclusion of additional parameters and alternative techniques for determining species specificity (e.g., molecular genotyping) within a comparative approach will allow insights into factors of bloom dynamics. Finally, incorporation of bio-optical patterns and phytoplankton datasets into local and regional oceanographic models will lead to improved forecasting and surveillance for HAB events.

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AFLP reveals intraspecific variations in geographically diverse *Ostreopsis* cf. *ovata* populations

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Abstract

Ostreopsis ovata is a species-complex harbouring several cryptic species. The Mediterranean-Atlantic populations are homogeneous in terms of phylogenetic markers, but show differences in physiological and ecological properties among sub-regions of the area. The aim of our study was to set up and validate a genetic fingerprint method that can be applied to assess genetic diversity within and among populations of *Ostreopsis ovata*. To this end, we selected the AFLP (Amplified fragment length polymorphisms) technique. We successfully generated distinct AFLP fingerprints for a number of *O.* cf. *ovata* strains isolated from three different sites of the Italian coasts. Reproducibility was confirmed by very similar molecular profiles obtained from three replicates for each strain. A binary matrix was compiled and a cluster analysis of the clones was performed to build a dendrogram for the evaluation of the relationships among strains. Results of this first analysis show that strains from Ancona and Taormina (Adriatic and Ionian seas, respectively) are more closely related amongst themselves than with Neapolitan strains, and confirms AFLP as a powerful technique for the identification of *Ostreopsis ovata* populations at the regional scale.

Keywords: AFLP, Mediterranean Sea, *Ostreopsis* cf. *ovata*, population genetics

Introduction

Ostreopsis ovata Fukuyo (Fukuyo 1981) Fukuyo 1981) is a benthic and toxin-producing dinoflagellate that has notably increased in abundance, frequency and geographic range in temperate areas over the last decade. Recent studies have highlighted that several cryptic species are hidden in this taxon (Sato et al. 2011) Sato *et al.* 2011), which is a species-complex within which the actual *O. ovata* is still to be defined.

Within the species-complex, one dominant taxon, identified as *O.* cf. *ovata*, coexists with less represented genotypes (Penna et al. 2010) Penna *et al.* 2010). *Ostreopsis* cf. *ovata* populations from different coastal areas display marked differences in the bloom time, varying between early summer in the NW Mediterranean Sea and late summer-autumn in the Adriatic Sea (Mangialajo *et al.* 2011; Pistocchi *et al.* 2011; Totti et al. 2010) Totti *et al.* 2010). In addition, a smaller size and slight differences in cell shape were observed in Adriatic compared to Tyrrhenian strains (Guerrini et al. 2010) Guerrini *et al.* 2010). These differences could be the results of intraspecific variations and physiological responses to different environmental conditions. On the other hand, it is also possible that these distant benthic populations are genetically different. Based on phylogenetic markers such as the partial LSU rDNA and ITS rDNA region 5.8S rRNA, strains of *O.* cf. *ovata* are genetically uniform across the Mediterranean-Atlantic area (Penna et al. 2010) Penna *et al.* 2010). In addition, strains with identical ITS sequences have also been retrieved in Japanese coastal waters (Sato et al. 2011) Sato *et al.* 2011). However, whether genetic diversity still exists among natural populations of this taxon has not been addressed so far.

In this work, we used the AFLP (Amplified fragment length polymorphisms) technique to analyse monoclonal cultures of *Ostreopsis* cf. *ovata* of different geographic provenance. We also compared the resolution of the fragment analysis approach with results obtained from the sequencing of the internal transcribed spacer (ITS) of rDNA.

Material and methods

Eighteen monoclonal cultures of *Ostreopsis* cf. *ovata* were obtained from coastal waters of three different Italian sites. Eight Adriatic Sea strains were isolated during summer 2008 and 2010 from

Fig. 1. Map of Italy with the position of the three sampling sites.

Ancona coastal waters (courtesy of C. Totti). The two Ionian Sea strains were isolated in late summer 2007 and 2010 from Taormina waters (courtesy of M.G. Giacobbe). The eight strains from the Tyrrhenian Sea were isolated in summer 2011 at Rocce Verdi and Gaiola, two close stations in an embayment located along the northeastern coast of the Gulf of Naples.

AFLP markers were developed following the method set up for *Alexandrium tamarense* (John et al. 2004) John *et al.* 2004). Two AFLP experiments were conducted: the first included two strains from each of the three sampling locations, the second included 8 strains from Ancona and 8 from Naples. In both analyses, two enzymes were used to obtain genomic DNA restriction fragments: the frequent cutter *MseI* (New England BioLabs) and the rare cutter *EcoRI* (Roche). Four primer combinations, including specific enzyme restriction sites plus three additional bases (EcoRI + AAG, EcoRI + ACC, MseI + CTA , MseI + CTT), were tested to find the best band resolution. The PCR products, run on ABI Prism 3730 DNA Analyzer (Life Technologies), were sized with GeneScan TM -500 LIZ Size Standard (Life Technologies). Results were visualized as electropherograms in GeneMapper 3.7 software (Life Technologies). Analyses were conducted on DNA fragments between 50 and 500 bp length, with a peak height threshold of ca. 50 relative fluorescent units (RFU) and a bin width of 1.0 bps. Reproducibility tests among three replicates were performed by setting in GeneMapper the Advanced Peak Detection Algorithm with a sizing range 50-500 bps, in order to obtain a higher percentage of homology.

The presence/absence of each peak was recorded and binary data matrices were constructed. The results obtained with the GeneMapper software were also checked manually for each individual genotype. GenAlex 6 software (Peakall and Smouse 2006) Peakall and Smouse 2006) was used for assessing the number of total bands and percentage of polymorphisms obtained for each enzyme-primer combination. An UPGMA (Unweighted Pair Group Method with Arithmetic mean) tree were obtained with the software PAUP version 4.0 (Swofford 1998) Swofford 1998) by heuristic search. Statistical support of branches was tested by 1000 bootstrap replicates.

The ITS rDNA region, including ITS1, ITS2 and 5.8S rRNA genes, was amplified using the oligonucleotide primers ITSA and ITSB according to Sato *et al*. (2011) 2011). Sequences were obtained and compared using BioEdit version 7.1.3.0 (Hall 1999) Hall 1999).

Results and discussion

The ITS region sequence showed no variations among the strains analysed, despite their distinct geographic origin. The ITS sequences were also identical to other *O.* cf. *ovata* sequences from GenBank, including AB674903, AJ311520 relative to strains isolated from Tei, Kochi, Japan and Gioia Tauro, Calabria, Italy, thus confirming the genetic homogeneity of this taxon across its range, as already shown in previous studies (Penna *et al.* 2005; Sato et al. 2011) Sato *et al.* 2011).

In AFLP experiments performed on a subset of isolates, reproducible amplified fragment profiles were obtained from reactions using i) the same DNA extract (technical replicates), and/or ii) serially diluted DNA extracts obtained from the same isolates at different times and with different cell quantities (biological replicates). Reproducibility tests among technical replicates scored a homology higher than 90% while biological replicates showed a lower percentage (60%).

Biological replicates homology can be compromised by biases introduced during the analysis, such as PCR stutters, non-specific amplifications, differences in peak mobility and intensity in the fingerprint profiles,

causing a reduction in the signal-to-noise ratio and an overall loss of reproducibility (Meudt and Clarke 2007) Meudt and Clarke 2007). In our case, with the aim of obtaining optimal results and refining the methodological procedures, we tested different amount of input DNA for the restriction, as well as different amounts of fragmented DNA loaded in the capillary electrophoresis for the analysis. The biological replicate homology percentage was hence affected by results from different run conditions. In both experiments the best enzyme-primer combination was *Eco*RI-ACC/*Mse*I-CTA, which produced the highest number of total and polymorphic bands (Table 1).

Table 1. Diversity of the 16 *O.* cf. *ovata* strains based on 4 AFLP primer combinations. Best primer-enzyme combination in bold.

Enzyme-primer combinations	Total bands		Polymorphic bands		Polymorphism (%)	
Exp. no	**I**	**II**	**I**	**II**	**I**	**II**
***Eco*RI-AAG/ *Mse*I-CTT**	223	350	73	270	33.18	77.22
***Eco*RI-AAG/ *Mse*I-CTA**	230	377	74	290	32.03	77.06
***Eco*RI-ACC/ *Mse*I-CTA**	**290**	**381**	**106**	**303**	**36.68**	**79.79**
***Eco*RI-ACC/ *Mse*I-CTT**	306	380	94	295	30.83	77.89
Total	1409	1488	346	1158	-	-
Mean	262	372	85	290	32.38	77.99

The first experiment, where two samples from each of the three populations were considered, showed *Ostreopsis* cf. *ovata* isolates from Taormina and Ancona (Adriatic - Ionian Sea) clustering together, while the Gulf of Naples (Tyrrhenian Sea) isolates clustered in a separate clade. Both clades were supported by high bootstrap values (Fig. 2).

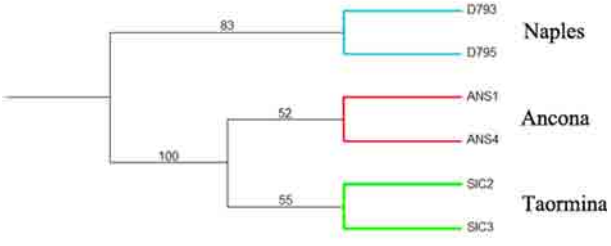

Fig. 2. UPGMA tree based on the AFLP binary matrix of the *O.* cf. *ovata.* Bootstrap values (1000 replicates) are indicated over the branches.

In the second experiment, where only samples from Ancona and Naples were included, strains from the two localities were also clearly distinct, with high bootstrap support (Fig. 3).

Conflicting results were hence obtained by using the two different approaches, ITS sequencing and AFLP. The rDNA ITS region has been demonstrated to be a powerful marker for detecting intraspecific differences across a wide range of species, including the unicellular organisms. This is the case, for example, of the pennate diatom *Pseudo-nitzschia multistriata*, where ITS analysis has revealed distinct sympatric populations in the Gulf of Naples (D'Alelio et al. 2009) D'Alelio *et al.* 2009). However, this phylogenetic marker is often homogeneous within species and can show cases of extreme resilience, as reported for *Scrippsiella hangoei* and *Peridinium aciculiferum*, where the ITS identity between the two species does not match the observed morphological and physiological differences (Logares et al. 2007) Logares *et al.* 2007).

A recent radiation *O.* cf. *ovata* in the Mediterranean/ Atlantic region has been suggested by Sato *et al.* (2011) based on ITS analysis, which showed little divergence and short branches in the Mediterranean/ Atlantic clade, as compared to the South China/ Malaysia/Indonesia clade, where a number of subgroups are clearly distinct. Our AFLP data, although preliminary, provide an indication that genetic differences may also exist among *O.* cf. *ovata* populations in the Mediterranean/Atlantic region. Divergence is probably recent though, thus not allowing enough time for neutral mutations to

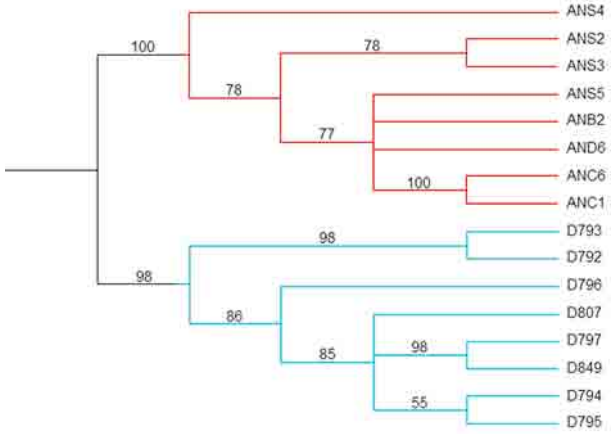

Fig. 3. UPGMA tree of *O.* cf. *ovata* inferred from AFLP data. Bootstrap values (1000 replicates) are indicated over the branches (red: Ancona, blue: Naples).

be fixed in the ITS rDNA of the distinct AFLP lineages. Indeed, as observed in other organisms (e.g. Bakkeren *et al.* 2000; Marhold et al. 2004) Marhold *et al.* 2004), by looking at different and larger areas of the genome AFLP allows to reveal genetic clustering at a much higher resolution as compared to ITS.

Conclusions

This study confirms AFLP as a sensitive, reproducible and effective technique for the study of population genetics and proves its applicability to the case of *Ostreopsis* cf. *ovata.* In contrast with the homogeneity showed by the ITS sequence comparison, AFLP indicates that *Ostreopsis* cf. *ovata* populations from different Mediterranean localities are genetically distinct. Deeper investigation using a larger number of isolates belonging to different locations is required to confirm these first results and clarify the population structure of *O.* cf. *ovata* in the Mediterranean Sea.

Acknowledgements

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A Regional Comparison of Upwelling, Coastal Land Use Patterns, and HAB Hotspots Along the California Coast

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Abstract

Blooms of harmful and toxic algae have increased in frequency and severity along the California coast during the past few decades. *Pseudo-nitzschia* is rapidly becoming the single greatest threat and problem for human and ecosystem health, due to the diverse impacts on the economy through commercial fisheries and tourism, as well as via direct impacts on marine birds and mammals. Our primary objective is to develop a better understanding of the ecophysiological conditions leading to bloom and toxin initiation for *Pseudo-nitzschia*, by simultaneously comparing two "hot spots", Monterey Bay and San Pedro, California. We hypothesize that large-magnitude blooms are associated with physical processes in California and that the large bloom events spanning hundreds of kilometers over a period of weeks to months initiate offshore or in the subsurface, allowing them to seemingly "appear" with little or no warning. There are a unique set of environmental conditions leading from bloom initiation to toxicity that can be identified through a comparative approach, allowing us to contrast potential factors between regions, and that blooms initiate both offshore and as subsurface layers.

Keywords: Domoic Acid; California, USA; nutrients; river discharge; anthropogenic discharge

Introduction

Spring blooms of toxic *Pseudo-nitzschia* are now a recurring problem along the U.S. west coast, first reported in central California during the 1990s and on an annual basis in southern California since 2003 (c.f. Scholin *et al*. 2000; Busse *et al*. 2006; Schnetzer *et al*. 2007; Anderson *et al.* 2008).
Many explanations have been proposed and investigated vis-à-vis the possible environmental driving forces for the observed patterns of harmful blooms in this region. The possibilities include hydrographic features that affect macro- and micronutrient availability (coastal upwelling, the proximity to canyons, the existence of retention/ incubation areas, river and sewage effluent discharge) and biological aspects of the algae (e.g. light and nutrient competition, vertical migration, mixotrophy). Notable similarities and some marked differences exist in the relative importance of these forcing factors between central and southern California that might provide valuable insights into the similarities and differences in HAB initiation, taxonomic composition and persistence. The same harmful algae (HA) species occur along the entire coastline yet the appearance of blooms appears to be staggered in time. While central California experiences a strong influence from the California Current and is strongly influenced by intense agricultural use of coastal land, southern California typically experiences milder coastal upwelling due to seaward diversion of the California Current and presence of the Channel Islands. Inputs of nutrients from land runoff in southern California also reflect contributions from and modification by the large urbanized expanses of this region. These influences are certainly tempered by interannual climate shifts such as ENSO and NPGO, among others (e.g. Kahru *et al*. 2009).

The primary objective of our project is to develop a better understanding of the ecophysiological conditions leading to bloom and toxin initiation for *Pseudo-nitzschia*, by simultaneously comparing two "hot spots", Monterey Bay and San Pedro, California. Better understanding of these factors will lead to improved understanding of how bloom dynamics change in response to shifting environmental conditions, why "hot spots" exist, and will ultimately provide improved monitoring, predictive modeling, and management decisions.

Monterey Bay is an open bay with a persistent cyclonic eddy circulation and the presence of a "bloom incubator" region in the NE corner (an upwelling shadow). The San Pedro shelf region has, since 2003, become one of the biggest wildlife intoxication hotspots in California, and is characteristic of the Southern California Bight, with retentive circulation and long residence times compared to the open coast (e.g. Monterey). Many bloom events in California "appear" suddenly, and the first manifestation of widespread toxicity is frequently the presence of intoxicated marine birds and mammals with no corresponding increase in toxicity or high cell abundances at shore-based stations (e.g. Scholin *et al*. 2000). These bloom events span hundreds of kilometers of coastline over a period of weeks to months and may initiate offshore, or in the subsurface where monitoring is sparse, allowing them to seemingly "appear."

We are testing three hypotheses (H) using a comparative approach: H1: blooms initiate as subsurface features (subsurface maxima) and eventually manifest as surface blooms. H2: blooms are predominantly the result of advective processes and retention in eddy-like circulation; subsurface maxima are less important. H3: there are a unique set of environmental conditions leading from bloom initiation to toxicity that can be identified through a comparative approach, allowing us to contrast potential factors (such as stratification, nutrient load, nutrient type) between regions. The alternate hypotheses are that HAB events in California are truly stochastic and/or the underlying ecophysiological responses are so complex or varied over modest geographical scales that relatively simple conceptual models cannot adequately explain bloom and toxin initiation. Here we focus on H3, and more specifically on the role of anthropogenic nutrient discharge as a potential trigger for toxicity.

Monterey Bay.

Lane *et al.* (2009) developed predictive logistic models of toxigenic *Pseudo-nitzschia* blooms in Monterey Bay, California, from a multi-project dataset. Models were developed for year-round (Annual model) or seasonal use (Spring and Fall-Winter models). The consideration of seasonality was significant: chlorophyll a and silicic acid were predictors in all models, but period-specific inclusions of temperature, upwelling index, river discharge, and/or nitrate provided significant model refinement. Predictive power for 'unknown' (future) bloom cases was demonstrated at ≥75% for all models, out-performing a chlorophyll a anomaly model.

When upwelling was directly evaluated as a predictor

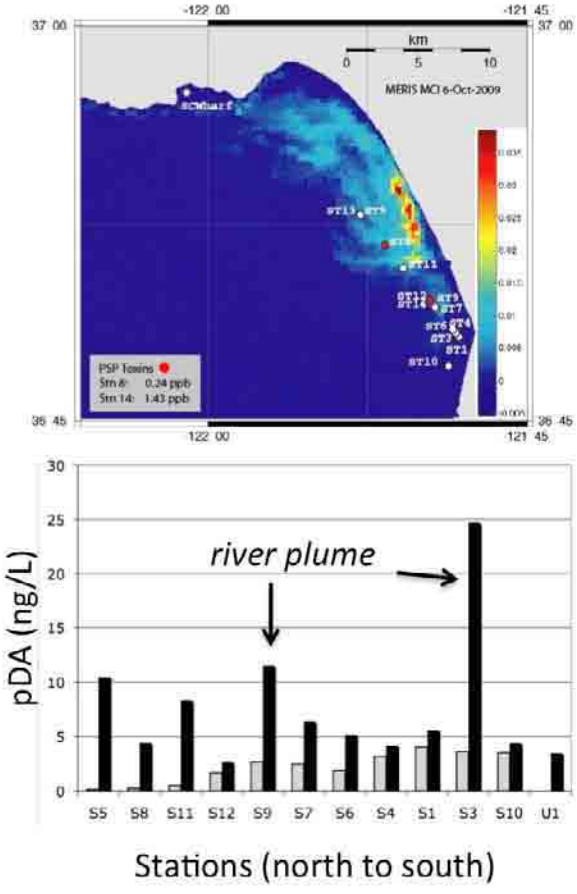

Fig. 1. MERIS maximum chlorophyll index (top) showing a large, nearshore bloom following the 2009 first flush event. Stations were occupied 2 days prior and 3 days post first-flush. Particulate DA (pDA) increased significantly at stations occupied before (grey) and after (black) the rain event, with maximum pDA associated with the river plume.

variable, a weak positive relationship was identified between upwelling and *Pseudo-nitzschia* bloom incidence throughout the year. Silicic acid and nitrate both emerged as significant predictor variables in models developed for *Pseudo- nitzschia* toxicity (Blum *et al.* 2006) and in the models developed by Lane *et al*. (2009). In both studies, the relational patterns agree: association to the dependent variable is negative for silicic acid and positive for nitrate. The Fall-Winter model from Lane *et al.* (2009) which addresses the time period in which 'first flush' (the first major rainfall event in the autumn) and high riverine discharge events generally occur, also demonstrates a direct negative relationship between river discharge and bloom incidence. Based on these model results, we expected riverine input of nutrients to be negligible drivers of toxin events.

Contrary to expectations, we have documented significant increases in domoic acid immediately following "first flush" events in 2009 and 2010. During both years *Pseudo-nitzschia* was present in subsurface layers (below surface dinoflagellate blooms) within the nearshore environment. Dissolved and particulate domoic acid increased significantly within days of the rainfall and subseqent fluvial inputs, with the largest increases associated with the river plumes (Fig. 1). Experiments manipulating salinity did not result in enhanced toxicity, but elevated concentrations of urea found in the river discharge have previously been associated with enhancement of *Pseudo-nitzschia* toxicity (Howard *et al*. 2007; Kudela *et al.* 2010); we did not directly test the role of urea in this study, thus the linkage remains speculative.

Southern California

In contrast to Monterey Bay, the Southern California Bight is unique because it is a region of some of the most intensive anthropogenic inputs along the U.S. west coast. The current population of southern California is more than 21 million and is expected to grow at a rate of about 1% per year exceeding 31 million by 2050 (State of California, 2007). Sixty point sources discharge over 4.7 million cubic meters of treated effluent per day into the coastal ocean (Lyon and Stein 2009)(Lyon and Stein 2009). The major point sources of pollutant discharge into the SCB are Publicly Owned Treatment Works (POTWs), power generating stations (PGS), industrial facilities, oil platforms, and dredged materials. Southern California POTWs contribute on the order of $3.6 \cdot 10^7$ Kg N/year (2.5 $7 \cdot 10^9$ M/year) to the coastal ocean. Two of the four major POTW discharges impact San Pedro Bay. In addition, following the approximately 35 annual storm events, runoff drains an area of over 14,000 km^2 and flows generally untreated to the SCB via storm drains, creeks, and rivers (Ackerman and Schiff 2003). We would expect that *Pseudo-nitzschia* would exibit strong responses to nutrient discharge in this region, analogous to the "first flush" response seen in Monterey Bay. However, there is little evidence for a direct connection between nutrient discharge and toxin events for the SCB (Schnetzer *et al*. 2007; Lewitus *et al.* 2012).

As part of a field program in September 2012, we monitored the coastal ocean during a planned diversion of the Orange County Sanitation District (OCSD) sewage effluent from a deep-water offshore pipe to a shallow pipe on the continental shelf. Conditions were calm and sunny, with significant discharge of nutrients and freshwater into the coastal ocean (ca. $6.75 \cdot 10^5$ $m^3 d^{-1}$ effluent). Despite the large input, a noticable decrease in salinity, increase in colored dissolved organic material and a drop in pH, there was little to no phytoplankton response. *Pseudo-nitzschia* was present and domoic acid was detectable, but there was no bloom and no increase in toxicity; more generally, phytoplankton biomass remained low throughout the ~21 days of the diversion.

Summary

We conclude that nutrient discharge into the coastal ocean can have unexpected and variable consequences. The interaction between nutrient discharge and HAB events is complex and there is not always a clear link between anthropogenic inputs and toxic blooms. The comparative approach is useful in teasing apart the underlying mechanisms driving these responses. Contrary to expectations, Monterey Bay exhibited a strong response to fluvial inputs (presumably in response to anthropogenic nutrients) while Southern California did not, despite the presumed importance of anthropogenic nutrients in Southern California and the presumed dominance of upwelling processes in Monterey Bay.

Acknowledgements

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Preliminary studies on HAB monitoring in the Persian Gulf and Oman Sea using remote sensing data from ocean color sensor MODIS

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Abstract

Remote sensing from satellite is a suitable way to observe surface waters phenomena. A red tide event occurred from early autumn 2008 until early spring 2009 in the Persian Gulf, Oman Sea and Strait of Hormuz. In this research, we utilized data of the ocean color sensor MODIS on satellite Aqua. With analysis of this data we made temperature, red tide index and chlorophyll-a images of the Persian Gulf and Oman Sea during the red tide event. Results of this research showed that lower water temperatures traced propagation of the bloom. High density blooms occurred mainly off populated industrial cities, and the main Persian Gulf current caused seemed to cause their transport to the western region. The highest bloom density detected by the satellite was observed in the northern Strait of Hormuz.

Keywords: *Cochlodinium polykrikoides,* ocean color teledetection, Persian Gulf and Oman Sea

Introduction

There are several adverse factors which threat the quality of environmental waters of the Persian Gulf and Gulf of Oman: increased urban (sewage) and industrial pollution, oil spills from oil wells and tankers, heavy metals and chemical loading from seaports located across the Persian Gulf coasts, and daily passage of over 18 million barrels of oil through the Strait of Hormuz.

A record harmful algal bloom (HAB), in terms of intensity and duration, occurred in the Persian Gulf and Gulf of Oman from autumn 2008 to spring 2009. The HAB event, caused by *Cochlodinium polykrikoides*, had devastating effects on fisheries, aquaculture, tourism and the environment. Precise analysis of the phenomenon, with qualitative and quantitative examination of its main causes, is needed to solve the problem. However, lack of awareness by public authorities from the Persian Gulf states and managerial inconsistencies have led to the inability of these countries to comply with environmental standards.

Efforts have been invested in different parts of the world to use chlorophyll and its detection by ocean color sensors to map regions affected by high density HABs (Ahn and Shanmugan 2006; Ishizaka 2003; Lavender and Groom 2001). Additional methods have been developed based on the unique, species-specific optical features of some HABs (O'Reilly et al 1998) and the use of classification techniques to separate HAB affected regions from other waters with distinct optical features (Pasterkamp et al., 2002).

In the present study, satellite MODIS was used to detect changes of marine physical parameters including ocean colour associated with chlorophyll a (chl-a), sea surface temperature (SST) and surface waters discoloration across the Persian Gulf, Strait of Hormuz and Gulf of Oman (Fig.1). Daily changes of these parameters were used to estimate changes

Fig. 1. Simplifed diagram of general circulation patterns in the study area (from Hamzehei et al 2013, based on Reynolds 1993)

in algal concentration during the red tide event, the extension of the affected areas and to analyze bloom transport pathways.

Material and Methods

Daily Level 1 and 2 MODIS Aqua chlorophyll a (chl-a) and sea surface temperature (SST) images were downloaded from NASA Ocean Color Web site (Fig. 2). Firstly, the imageries are processed for the Rrs(555nm) by using SEADAS software to identify the turbid waters where chl-a over-estimation problem occurred by the chlorophyll algorithm. Red-tide Index (RI) images are provided as the additional reference for the ocean color image. RI is based on the principle that red tide organisms absorb radiation in the green to blue wavelength while strongly reflects radiation in the green wavelength. The RI was defined as the ratio between [Lw(510)/Lw (555) − Lw(443)] and [Lw (510)/Lw (555) + Lw(443)] (Ahn and Shanmugam, 2006).

Results and Discussion

Initiation, development, spreading and decline of the 2008-2009 algal bloom in the Persian Gulf region are reported here. Growth conditions for *C. polykrikoides* blooms in Korea were defined by Kim *et al*. (2004): temperature of 21 to 25 °C and a huge salinity range of 15 to 50 psu. However, bloom increase even when temperature was 29 °C was observed in the Persian Gulf and Gulf of Oman. This record bloom persisted during 10 months at the northern Strait of Hormuz. The bloom mechanism by which *C. polykrikoides* persisted for such a long time in this area remains unclear, but one of the reasons may be its high temperature tolerance (Hamzehei *et al.* 2013).
Supply rates of organic matter in coastal waters off industrial zones of Bandar Abbas and Qeshm, northern Strait of Hormuz, are very high (Hamzehei 2007) and failure to treat urban sewage and industrial waste in this area have been suggested to trigger the high algal densities persisting during a 10 month period. The high density algal patches appeared periodically transported towards other areas. This was very clear in the northern and southern parts of the Gulf of Oman.
Satellite images showed SST dropped below 27 °C (due to upwelling) and high values of ocean color in the red tide areas (Fig. 3). Color images suggested

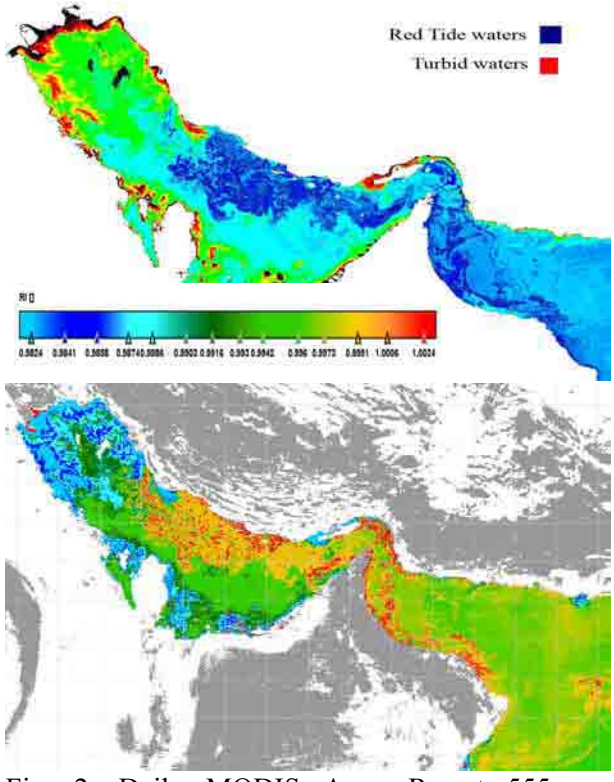

Fig. 2: Daily MODIS Aqua R_{rs} at 555 nm associated with red tide and turbid waters (top) and RI (bottom) on 21 December 2008

the algal densities of the red tide were high off overpopulated cities by the Strait of Hormuz, such as Bandar Abbas and Qeshm and that the water inflow into the Persian Gulf appeared to be effective in stretching the red tide to the more western regions of the Persian Gulf.
The most striking patch detected by satellite was located along the northern Strait of Hormuz, and survived there for more than 9 consecutive months. Stretching of the patches paralleled the current flow patterns described for the Persian Gulf and Oman Sea (Hamsehei 2007; Hamsehei et al 2013; Reynolds 1993). Oceanic mesoscale eddies may have played an important role in the Gulf of Oman transporting algal patches from southern to central and northern regions. Shortage of nutrients may have been suggested to cause the bloom decline in the Oman Sea.
Meteorological conditions, and species-specific nutrients and biological constraints might explain the long term persistence and decline of the bloom. Nevertheless, detailed in situ measurements and numerical modeling, carried out in cooperation with neighboring countries of the region, would be

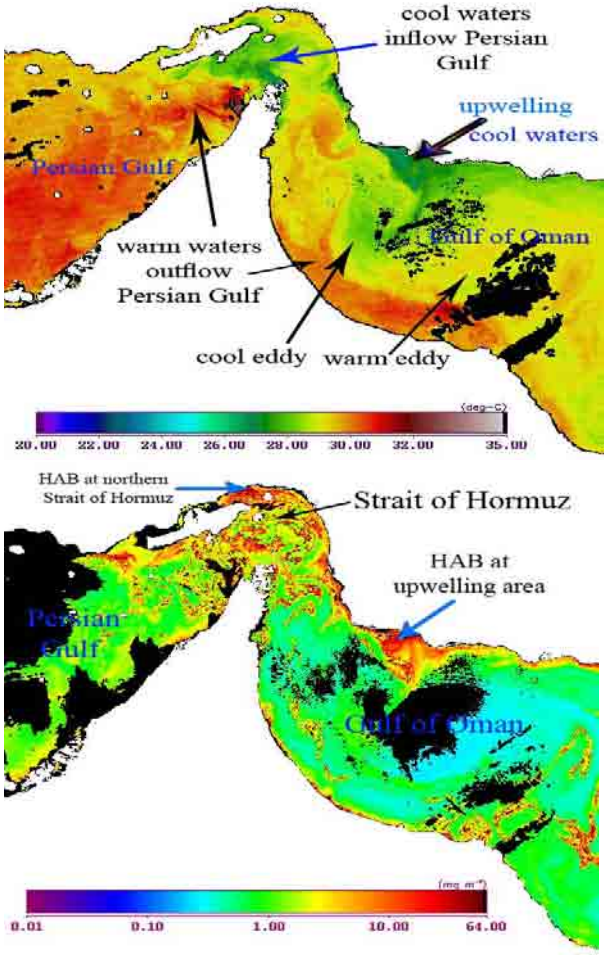

Fig. 3: SST (top) and chl-a (bottom) of MODIS Aqua on 31 October 2008.

required to test different hypotheses and understand bloom mechanisms in the Persian Gulf and Oman Sea.

Conclusions

Highest values of ocean color associated with the bloom were located along the northern Strait of Hormuz, where these values persisted for more than 9 consecutive months. The extension of the patches followed the general current flow patterns in the Persian Gulf and Sea of Oman. Oceanic mesoscale eddies may have played an important role in the transport of algal patches from southern to central and northern parts of the Oman Sea. A better knowledge on temperature conditions (meteorology), nutrients and species-specific biological constraints is needed to explain the long term persistence of *Cochlodinium polykrikoides* blooms in the Persian Gulf and Oman Sea

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Risk Stratification of Paralytic Shellfish Poisoning (PSP) in Shellfish of the Gulf of Thailand

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Abstract

A Receptor Binding Assay (RBA) was used to estimate Paralytic Shellfish Poisoning (PSP) toxin in shellfish from the Gulf of Thailand and the Andaman Sea. The distribution pattern of PSP toxins was assessed through the PSP toxicity level in 265 shellfish specimens belonging to 5 distinct species. *A. granosa* was rated as a the highest risk species (risk1) among all the samples. Risk stratification within the Gulf of Thailand and the Andaman Sea indicated that the Eastern Gulf of Thailand is the most susceptible area to PSP incidents. PSP toxicity levels did not show significant variation with season. The toxicity levels in fish species were found to be highest in March and November with 18 and 12% fish rated, respectively, as frequent poisoners. However, the risk ranges were still at low-risk zone (risk0). Moreover, the total number of individuals sampled in November were too small (n=8) to be of real representative value for this time of the year.

Keywords: Paralytic Shellfish Poisoning (PSP), shellfish, risk, Gulf of Thailand, Andaman Sea

Introduction

Paralytic shellfish poisoning (PSP) poses a serious health hazard to humans, including human fatalities after eating contaminated shellfish and economic losses due to closures of shellfish harvest (Bricelj &Shumway 1998; Zhou 1999). Thailand is a country highly dependent on its marine environment for food, raw materials and other resources for subsistence and economic development. However, a national monitoring program for risk assessment for PSP in fish and fish products has never been performed, possibly due to the lack of a suitable method for monitoring a huge amount of samples with low toxin contents. Recently, a Receptor Binding Assay (RBA) method (Doucette et al. 1997; Powell & Doucette 1999) has been used as a fast and sensitive alternative to the Mouse Bioassay (MBA) in laboratories around the world. The RBA for PSP toxins has been evaluated in an AOAC single laboratory evaluation (Van Dolah et al. 2009) and has been approved as an official analytical procedure of the AOAC for PSP determination following an AOAC Inter-laboratories trial (Van Dolah et al. 2012). In this study PSP toxins in 265 shellfish specimens belonging to 5 species, from 5 regions of the Gulf of Thailand and the Andaman sea, were analyzed with a RBA method. The objectivev was to asses toxin distribution pattern and risk stratification in the region.

Material and Methods

Shellfish samples were collected in 5 areas of the Gulf of Thailand and the Andaman Sea (Fig. 1). The shellfish species collected at each site are shown in Table 1. About 50 to 70 mm averaged-size whole mussels were collected and cleaned with seawater. Sampler mussels were kept and transported to the laboratory, where each mussel was measured, their shell removed and the flesh weighed. Flesh was homogenized with a blender, and 200 g of homogenized sample was put in a zip bag and kept frozen at -80^{o}C until analysis. Physico-chemical data such as DO, pH, salinity, water temperature, water depth and transparency were measured and recorded.

The shellfish extract was subjected to the Receptor Binding Assay. The assay quantifies toxin activity through competitive binding (to mouse membrane receptors) of unlabelled toxin in the sample and known quantities of radiolabelled saxitoxin ([^{3}H]STX).

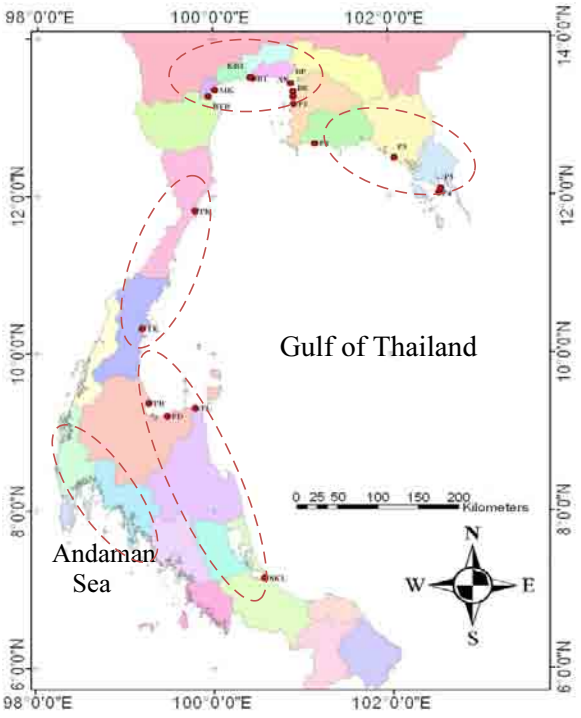

Fig.1 Map of Gulf of Thailand and the Andaman Sea showing sampling locations.

A complete description of the assay is given by Srisuksawad et al (2010). In brief, five brains (with medulla removed) of male, wistar, six week old mice (National Laboratory Animal Centre, Mahidol University THA) were grounded. In per one brain, 12.5 ml of ice-cold homogenization buffer; 100 mM MOPS, pH 7.4 (Bio Basic 1132-61-2, ultra pure USA), 100 mM choline chloride (Sigma C7527-200g USA), 1 ml of 0.1 M PMSF (Phenyl methylsulfonyl fluoride) (Sigma P7626-100g USA) using a Teflon/glass tissue homogenizer (Glas-Col® 099C K6424 USA). Individual preparations were then pooled and re-homogenized on ice. The tissue homogenate was centrifuged at 20000 g for 15 min at 4°C and the supernatant discarded. The pellet was resuspended in 50 ml homogenization buffer and treated with a Polytron tissue homogenizer at 70% maximum setting for 20 sec on ice. A sample was removed for determination of protein content and the remainder divided into 1 ml aliquots and stored at -80°C.

To generate a standard curve, unlabelled Saxitoxin (STX) was diluted with distilled water in half log dilutions over a range of (value are prior to dilution in the assay): $6X10^{-6}$, $6X10^{-7}$, $1.8X10^{-8}$, $6X10^{-8}$, $1.8X10^{-8}$, $6X10^{-9}$ and, $6X10^{-10}$ M. The assay was performed on a 96-well microtiter filter plate (Millipore MAFC NOB 50 USA). In triplicate, 35µl of each dilution was added to 35 µl of 15 nM [^{3}H] STX in ice-cold choline chloride binding Toxin quantification: Three separated dilutions of the unknown sample were prepared in distilled water. The assay was performed as the above while preparing the Standard Curve. The quantity of STX equivalent was obtained by solving the regression equation for X from the standard plot.

Risk rank definition: At the individual level, PSP risk was assessed following Chinain et al. (2010). In summary, each shellfish species was arbitrarily assigned a risk range based on its RBA values: "(-)": RBA< 0.31 ng STX equiv. g^{-1} or 3.1 µg STX equiv.$100g^{-1}$, "(+)": RBA = 0.31-0.36 ng STX equiv.g^{-1} or 3.1-3.6 µg STX equiv.$100g^{-1}$,"(++)": RBA = 0.37-0.8 ng STX equiv.g^{-1} or 3.7-8 µg STX equiv.$100g^{-1}$,"(++)"; "(+++)": RBA = >0.8 ng STX equiv.g^{-1} or <8 µg STX equiv.$100g^{-1}$. At the species level, a given species group was regarded as generally safe/infrequent poisoner when at least 70% of tested specimens were rated (-) or (+) and, conversely, was regarded as a frequent poisoner when at least 70%of individuals were rated (++) or (+++). At the spatial level , the PSP risk associated with each sampling area was defined by a range

Table 1 List of geographical origin of shellfish species sampled in study areas and number of individuals tested per species

Shellfish species	Common name	Local name	Sampling areas[a]	N[b]	Percentage of shellfish per total samples
A. granosa	Blood cockle	Hoi krang	A,C	17	5.6
P. veridis	Green mussels	Hoi malang pu	A,B,C,D,E	141	53.7
A. gigas	Pacific oyster	Hoi nang rom	A,B,C,D	84	32.1
M. meretrix	Oriental hard clam	Hoi talab	A	13	4.9
S. regularis	Razor clam	Hoi lort	A	10	4.1

Note: [a] for the completion of the definition of the area sampled: A=Upper Gulf of Thailand; B=Eastern Gulf; C=Central Gulf; D= Southern Gulf and E= Andaman Sea. [b] number of samples absence or low risk of PSP, i.e. > 70% of the shellfish tested in that area were rated (-) or (+),

from 0 to 2, where 0 means with no more than 20% of fish in the (+++) group, and 2 maximum risk, i.e. presence of toxic blooms and > 70% tested in that area rated (++) or (+++).

Results and Discussion

Shellfish was assayed for PSP toxins in 265 shellfish specimens of 5 species collected from 5 areas of the Gulf of Thailand and the Andaman Sea. Some species were not found in all areas. For example, *M. Meretrix* and *S. regularis* were found in the upper Gulf and *A. granosa* in the upper and central Gulf only (Table 1). Table 2 presented the respective percentage of individuals per species that were rated based on PSP values. The highest PSP values were 13.10 and 7.22 µg STX equiv./100g flesh and were reported in *A. granosa* and *P. veridis,* respectively. The shellfish species most prone to PSP in Gulf of Thailand were blood cockle *A.granosa,* for which all 15 specimens tested were positive, followed by the green mussel *P. veridis* (n= 141), the pacific oyster *C.gigas* (n=84), the oriental hard clam *M. meretrix* (n=13), and the razor clam *S. regularis* (n=10).

Table 3 shows the overall distribution of the 265 specimens analyzed per sampling area and per risk category. From these data, the proposed risk stratification within the Gulf of Thailand and the Andaman Sea was the following: Eastern Gulf of Thailand is the most susceptible areas to PSP incidents (risk 1) with 46% fish rated as frequent poisoners/high-risk specimen. The southern Gulf and the Andaman Sea appeared to be low risks zones (risk0) with 100% of fish rated as safe/infrequent poisoners for both. Of note, all *P. veridis* individuals were found consistently toxic whatever their sampling area. This species displayed the highest values in 4 of the 5 studied areas.

Seasonal variation of PSP risk is shown in Fig 2 which presents the respective percentage of individuals per sampling time rated (-) to (+) versus (++) to (+++), based on RBA values. PSP risks did not show a distinct seasonal variation. The highest risk ranks were in March and November with 18 and 12% fish rated as frequent poisoners. Although the risk range was still at the low−risk zone (risk0), the total number of individuals sampled in November were too small (n=8) to be representative for this time of the year.

Conclusion

The risk stratification of PSP in the Gulf of Thailand and the Andaman Sea was assessed through the toxicity level in 265 shellfish specimens belonging to 5 different species. The highest PSP values were found in *A. granosa* followed by *P. viridis* (13.10 and 7.22 µg STX equiv.100g $^{-1}$flesh), respectively. *A. granosa* was rated as a high-risk species (risk1). A map of the risk stratification within the Gulf of Thailand and the Andaman Sea indicated the Eastern Gulf is the most susceptible area to PSP incident. PSP values did not show a significant variation with season. The value of fish species were found to be highest in March but still the risk range was low (risk0) and the total number of individual sampled in November were too small (n=8) to be representative.

Table 2. Shellfish species samples in Gulf of Thailand and the Andaman Sea and variation in toxicity levels among the specimens as assessed by RBA

Fish species	N[a]	Percentage of fish species of each category [b]				RBA Values			PSP risk [c]
		(-)	(+)	(++)	(+++)	min	max	area	
A. granosa	17	53	6	35	6	0.98	13.10	Upper Gulf	1
P. veridis	141	96	-	4	-	<0.24	7.222	Central Gulf	0
C.gigas	84	98	-	2	-	<0.24	4.628	Upper Gulf	0
M. meretrix	13	100	-	-	-	<0.24	1.766	Upper Gulf	0
S. regularis	10	100	-	-	-	<0.24	0.897	Upper Gulf	0

[a] Total number of fish specimens sampled and tested per species

[b] Risk rank code for fish specimens: (-):RBA< 3.1; (+):RBA=(3.1-3.6); (++):RBA=(3.1-8);(+++):RBA>8 : RBA values are expressed in µg STX equiv. .100 g^{-1} shellfish.

[c] Risk range code for shellfish specimens : 0: non toxic or low-risk zone ; 1: medium toxic- risk zone ; 2: high-toxic risk zone

Table 3. PSP risk stratification within the Gulf of Thailand as inferred from toxicity analysis conducted on 265 shellfish specimens using the RBA

Sampling area	N[a]	Percentage of shellfish species of each category				RBA Values			PSP risk
		(-)	(+)	(++)	(+++)	min	max	species	
Upper Gulf (A)	100	90	1	8	1	<0.24	13.10	*A. granosa*	0
Eastern Gulf (B)	93	48	5	46	-	<0.24	5.42	*P. veridis*	1
Central Gulf (C)	23	91	-	9	-	<0.24	7.22	*P. veridis*	0
Southern Gulf (D)	23	100	-	-	-	<0.24	2.65	*P. veridis*	0
Andaman Sea (E)	14	100	-	-	-	<0.24	2.51	*P. veridis*	0

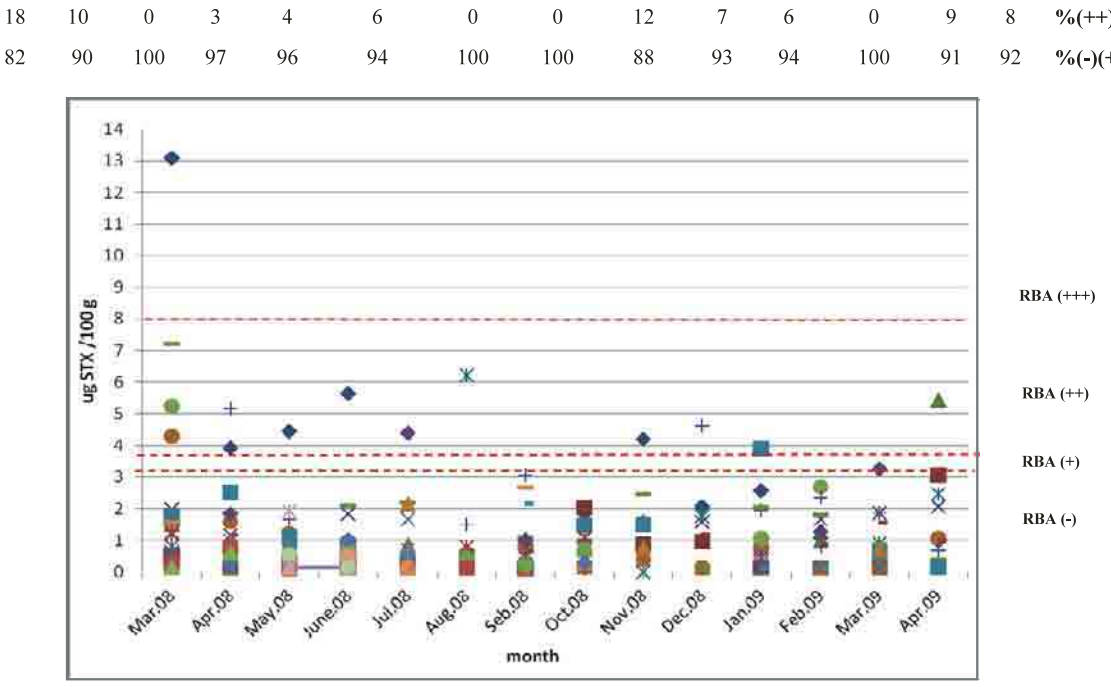

Fig. 2 Seasonal variations in toxicity levels among specimens, as assessed by RBA. Top two lines inferred the percentage of shellfish specimens of each category.

Acknowledgements

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Phytoplankton bloom strategies and constraints on predicting outbreaks

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Abstract

The appearance of high biomass algal blooms including harmful ones is frequently both sudden and unanticipated, and their duration is normally brief. These characteristics have historically frustrated detailed observation as well as theoretical understanding. Monitoring programmes are now relieving the first problem, but modeling and prediction still present major challenges. Predictions are of course blurred by historical contingency, but several other brakes probably also contribute to slow progress. These include i) a general focus on vegetative growth to the exclusion of other life history stages, ii) a view that division rates and other traits are fixed parameters, while phenological adaptations are of no consequence, and iii) over simplification of trophic links and other forms of biological interactions that affect population dynamics.

Keywords: fitness, life-cycles, extended dormancy, population dynamics

Introduction

The *fundamental particles* of many models of phytoplankton dynamics are isolated cells which divide as rapidly as resource supplies allow. The cells possess neither memories nor life histories, do not have resting phases, exhibit neither social nor sexual activities: all population regulation is extrinsic. Organisms are the ultimate victims of happenstance (there are no inborn filters or templets), and live in a perpetual state of *tabula rasa*, like Java applets. Population dynamics is then a simple accountancy problem in which division rates and loss rates determine particle abundance, and phytoplanktonic life is an exponential progression constrained only by diffusion and death, a pure Malthusian existence. We take the view that all species have life-history patterns controlled by evolution, which in some cases include extended dormancy as resting stages, or prolonged no-growth phases.

Earlier accounts of red tides frequently emphasized two features, i) that they do not occur with any regularity, often only being reported at long time intervals (several or many years) − the variance spectra of abundance are red; ii) the sudden occurrence of high cell concentrations, and their ephemeral duration, lasting only a few days.

We suggest there is a spectrum of strategies which ensure long lasting survival in highly variable environments, and that account for these two features. These include control of vegetative phase abundance (N^*) at levels lower than nominal carrying capacity (K), achieved by cell signaling (quorum sensing), tactical means to minimize loss rates while achieving high abundance, and delayed excystment schedules equivalent to metazoan age-class structures. These adaptations point to the existence of targets for natural selection additional to the classical *r*- and *K*- targets of MacArthur and Wilson (1970). In addition, considering the interplay among different adaptive responses, rarely can trade-off options be arranged in a linear fashion.

Fitness

To achieve darwinian success, an organism must out-compete other members of the local population, and avoid whatever hinders that aim. It must do better than average to stay in the game. The standard measure of this kind of success, called *fitness*, is Fisher's (1958) 'net reproductive value' (r), the Malthusian parameter of genetics, the intrinsic rate of natural increase in demography. For a particular genotype, r is the mean number of successful progeny produced *over the life cycle* (not μ, the division rate over the growth phase); for a population, the net reproductive value is $(r - m)$, where m is the loss rate, and depends on other phenotypes in the population and on the environment. Thus $r = [lim/t \rightarrow \infty][\log (N_t) - \log$

$(N_0)]/t$ while $\mu = [\log (N_t) - \log(N_0)]/t$.
Theory tells us that over a single generation, or in a constant environment, selection should seek to maximize *r*; but over many generations and in variable environments, this is not an optimal strategy (Cohen 1966; Wyatt and Jenkinson 1997). The optimal times to switch between life history stages such as excystment and encystment depend on the *mean* deviations of the season in which vegetative growth is possible, not on its length. These optima cannot therefore be triggered by ambient conditions, but must depend on an endogenous mechanism or clock. If excystment is spread across future growth opportunities to introduce delays in a life cycle, a form of bet-hedging, i.e. risk-spreading strategy (Beaumont *et al.* 2009), is achieved. In species that do not form resting stages, the same strategy is realized through (diversification of) clocks regulating the transitions between growth and no-growth phases (D'Alelio *et al.* 2010). The spreading reduces variance in expected fitness, which in turn maximizes mean fitness in an uncertain environment. So, most is not always best. Focus here is on the demography of phytoplankton with complex life-cycles in variable environments – we return briefly to the genetic aspect later.
Normalized by longevity, the two characteristics of apparent irregularity and ephemerality are typical of many pelagic organisms, not just some phytoplankton species, including some commercial nekton. These features suggest we are witness to specialized demographic strategies. In fact we can recognize a spectrum of life-history strategies (Lewis 1977; Zingone 2002) which are reflected in increasingly complex patterns of appearance of the planktonic phase (Wyatt 2014).

Elementary life histories

Simplified models of some dinoflagellate life-histories contains two alternating forms, an actively dividing planktonic vegetative stage and a benthic dormant cyst stage; *transitional* forms such as gametes and planozygotes usually have no rôles in such models; they are in any case generally more fleeting, though planozygote are often long-lived. The two (or more) stages must be coordinated, like the somatic and reproductive tissues of metazoans. Although we generally think of many species that fit this model as planktonic, we can equally well visualize the cysts as sedentary 'adults' with a planktonic reproductive stage. In some species, dormancy of the cyst is prolonged or extended, in the sense that excystment does not necessarily take place at the next appropriate opportunity. The resulting excystment schedules presumably evolve within clones, since Hamilton's rule (1964) would otherwise preclude a gain in fitness. Different life history stages can respond independently to different selection forces.

Vegetative phase

The conventional rôles of the vegetative stage are acquisition of resources and growth, like applets. But this stage also leads to gametogenesis. If the resulting zygotes are to mature and become dormant cysts, then a further role of the vegetative cells is to provide them with the stores required. These activities obviously divert resources from growth, and mean that resource requirements exceed the amounts calculated on the basis of growth rates. Syngamy implies the need for communication, presumably mediated by chemical signals (like quorum sensing in bacteria).
The 'return time' or characteristic time scale of the vegetative phase depends on the division rate, and is estimated by $1/\mu$. Rapid bloom formation is an advantage to offset losses due to grazing and diffusion, so there is a trade-off between rapid growth and the need to set resources aside for other vegetative roles. Rapid return times offer the possibility of rapid shifts between alternative population equilibria. Planktonic cells are generally thought to be dispersed and advected passively by the local hydrodynamic regime. This is an oversimplification (Squires 1996). Dispersion can be an alternative to dormancy in variable environments, but a degree of population viscosity is necessary for genetic reasons (Waples 1998). Without it, outbreeding would overwhelm inbreeding. It is also argued that the evolution of sex is impossible in 'infinite' populations (Otto 2009). Mechanisms to achieve population viscosity in phytoplankton (vertical migration, thin layer formation, local environmental engineering) in turn imply the probability of coupling between local production by the vegetative stage and recruitment to nearby cyst banks. The relative inoculation rates of local and distant vegetative populations by local and distant cyst banks determine the extent to which metapopulation units are closed or open, hence estimate this viscosity.
The scale of population viscosity in phytoplankton is hardly known. Other plankton groups provide evidence that it could be much less than intuition

suggests, i.e. between < 10 and 50 km (with normal or exponential dispersal functions) for some fish larvae whose planktonic phases last much longer than the vegetative phases of many phytoplankton species (e.g., Paris *et al.* 2007, Puebla *et al.* 2012). In a *purely* diffusing world (no return circulation), automata cannot get *home* again with any reasonable probability. But in an advective system, which necessarily has a return route, they may be able to do so if they can find that route and get the return timing right; at that point they cease to be automata and can follow trajectories which are not determined by hydrodynamics, i.e., they can navigate. Some planozygotes may play a part in this context. But if r is high enough, they may not need to; then there is a trade-off between r and navigational ability.

Bloom formation

To colonize a water body and bloom, the reproductive value of the *vegetative phase* ($\mu - m$), where μ is division rate and m mortality, must be > 1. At K (carrying capacity), ($\mu - m$) = 1, i.e., if m is constant, μ declines as the fraction of the water body available for colonization declines. The mechanism of density-dependence is not generally known. These dynamics assume homogeneity while $N < K$ (where N is maximum population), while K is equated with crowding and taken to be detrimental (MacArthur and Wilson 1970). The partitioning of resources between several activities, trade-offs between life-history stages, and the 'threat' of K or other conditions unfavorable for continued growth indicate the need to anticipate changes in external conditions, and enter transitional stages with appropriate timing. These factors are likely to simulate uncoupling between growth and environment. For some species, it may be necessary to reach high cell densities (N*) so that life-history transitions can proceed. Formation of thin layers provides one route to this goal which is independent of division rate.
Grazers and parasites present barriers to a population's ability to reach N*. Obviously N* must be less than K, and is subject to natural selection. When bloom status is achieved, it may be necessary to optimize receptor allocation, and trade less nutrients for lower infection rates by viruses (Menge and Weitz 2009).

Cyst phase

We now turn to the rôles of dormant cysts. Wall (1975) listed five functions of the cyst stage of dinoflagellates, three directly linked to the population dynamics of the planktonic phase. These were to inoculate the water column, to control the timing of inoculation, and to aid dispersal. The last is unclear. The fourth function was genetic recombination, already accomplished in the model considered here in the planktonic phase, and the fifth to survive unfavorable conditions. The fifth is sometimes the only rôle recognized for dormant stages: in fact it mixes two rôles, to overwinter, and to cope with environmental variability. From the perspective of the cyst bank, the essential need for long-term survival is that it replenishes itself; this is achieved by sending colonists into the water column to initiate the vegetative phase. If inoculation is successful, as we have already described, it will eventually provide new recruits to the cyst bank (Eilertsen and Wyatt 2000; Estrada *et al*. 2010).
Excystment is a risk since conditions may prove unsuitable for growth. Extended excystment schedules spread the risk thus buffering environmental variability. If the environment simply fluctuates between good and bad, then the optimal germination fraction is equal to the probability of a good year. There is then a trade-off between the mean and the variance in the traits/fitness equation, meaning that individuals or clones with a higher mean trait related to fitness are not necessarily favoured. In a sense then, cyst banks and their excystment schedules can be compared to the iteroparous strategies of many fish.

Power spectra

If we plot the variance of biological census data on frequency (both logarithmic), the slope of the correlation can range from negative to positive. By analogy with the spectrum of visible light, these are known as red and blue; a zero slope in which all frequencies are represented equally is called white. Thus in the language of time series, red tide census data have red spectra, meaning the temporal variance in abundance is concentrated at low frequencies. It is especially typical that species which have cysts in their life-cycles have red spectra (Zingone *et al*. 2010). Red noise can be visible in populations with iteroparous reproduction but not in those with semelparous reproduction (Kaitala and Ranta 2001). We can call these phytoplankton with extended dormancy *seculoparous*.
Deterministic models of population dynamics do not

give red spectra, except with restricted parameter values, but model spectra can be reddened by adding e.g., delayed density-dependence, age-structure, life-cycles, and environmental variability. Since both geophysical and census data have red spectra, we can ask whether the former are responsible for the latter, i.e., is there a causal relation between climate and census. The answer is certainly yes, but statistical support is so far scarce for phytoplankton. We may nevertheless agree that "organisms that place the survival of certain life-cycle stages directly at the mercy of the ocean-atmosphere system may automatically acquire red-noise variability in their population levels" (Bakun 1966), with the reward that their availability to enemies is unpredictable.

Conclusions

The dynamical behaviour of populations is the sum of effects due to events with different frequencies (weather, hydrodynamics), but in sum red, in combination with endogenous regulation. Social control of population densities, of the timing of life-history transitions, and means to ensure some degree of population viscosity, are amongst these endogenous mechanisms. Life-cycles with extended dormancy allow reduced variance in expected fitness, which is equivalent to maximizing mean fitness in variable environments. Delayed dormancy strategies should hence be incorporated into models of phytoplankton dynamics.

O' Dor's *Law of Biology* states that chemistry and physics make the laws, and organisms seek out and exploit the loopholes in those laws. We can parody this law and say that modellers make the rules and organisms seek ways to confound these rules.

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Population dynamics of Harmful Algal Blooms including climate changes

Ocean acidification will not deliver us from *Ostreopsis*

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Abstract

Ocean acidification caused by increasing atmospheric CO_2 concentrations could influence benthic communities, including harmful microalgae living therein. With the aim of assessing the possible effects of pH decrease on benthic harmful algal blooms (BHABs), the distribution of *Ostreopsis* cf. *ovata* was investigated at volcanic CO_2 vents of the Ischia Island (Tyrrhenian Sea, Mediterranean Sea). Samples were collected on 5 sampling dates from the brown alga *Dictyota dichotoma*, at 6 stations located along marked pH gradients (6.8 - 8.1), of which 3 at a sheltered and 3 at an exposed site. *Ostreopsis* abundance was significantly higher in the exposed site ($<7.8 \pm 2.4 \cdot 10^5$ cells g^{-1} fw, July 2011) than in the sheltered one ($<7.4 \pm 3.9 \cdot 10^4$ cells g^{-1} fw, July 2011), but did not vary significantly along the pH gradient within each site. These results indicate that *O.* cf. *ovata* is tolerant to a wide range of pH values, while it is affected, in terms of abundance, by hydrodynamic factors and/or by their effects on the benthic biota. Apparently, ocean acidification is not likely to represent a hindrance to the spreading and intensification of *Ostreopsis* blooms, while, a relevant rôle could be played by hydrographic variations in coastal environments.

Keywords: *Ostreopsis*, ocean acidification, harmful algal blooms, benthic ecology

Introduction

Ostreopsis are benthic dinoflagellates which grow on macrophytes or other biotic and abiotic substrates. Species in the genus produce palytoxin-like molecules, which can provoke severe and also lethal intoxications in humans (Tubaro *et al.* 2011). In the last decade the geographic range, intensity and frequency of *Ostreopsis* blooms have increased markedly also in temperate areas (Parsons *et al.* 2012). At the same time, increased atmospheric CO_2 concentration is causing ocean acidification. It has been predicted that by 2100 atmospheric CO_2 will almost certainly double that of pre-industrial levels, lowering pH by 0.4 units (Caldeira and Wickett 2005). Multiple experiments show that ocean acidification will likely cause declines in calcification rates in marine calcifying organisms (Doney *et al.* 2009; Guinotte and Fabry 2008) affecting also rocky benthic fauna by simplification of community structure due to a different degree of vulnerability of echinoderms and molluscs (Hale *et al.* 2011). At the opposite side, primary productivity of the oceans could be enhanced by high levels of CO_2 (Riebesell et al., 2007). Nevertheless, the complex interplay of several factors influenced by climate variations may produce unforeseen impacts on the marine biota (Feng *et al.* 2008). Global climate variations and the increasing number of new algal bloom episodes is now adding a new level of uncertainty to many HAB monitoring programs (Hallegraeff 2010).
To assess the possible effects of ocean acidification on the toxic dinoflagellate *Ostreopsis* cf. *ovata,* we sampled the species along a pH gradient in a natural CO_2 venting site that we used as a field laboratory.

Study site

Castello Aragonese (40° 43.84' N, 13° 57.08' E) is a castle built on a rock ca. 300 m far from the north-east coast of Ischia Island (Tyrrhenian Sea, Mediterranean Sea), joined to it by a bridge. The site harbours volcanic CO_2 vents bubbling at about $1.4 \cdot 10^6$ l d^{-1} (Cigliano *et al.* 2010). Significant differences in pH are recorded in the area, where two sets of three stations each, about 100 m from one another, have been identified along two symmetric acidification gradients (Hall-Spencer *et al.* 2008). These gradients are positioned at two adjacent sites which are physically separated by the bridge connecting the castle to the island (Fig. 1). Dramatic effects of the low pH have been demonstrated on a

broad range of organisms in the area, including calcareous epiphytes (Martin *et al.* 2008), benthic diatoms (Porzio *et al.* 2012), whole macroalgal community (Porzio *et al.* 2011) and invertebrates (Kroeker *et al.* 2011). Although confined to small areas and subject to high variations in pH, these sites can provide information about the ecological effects of long-term exposures to acidified conditions and evaluate feedbacks and indirect effects that occur within natural marine systems (Hall-Spencer *et al.* 2008) (Riebesell 2008).

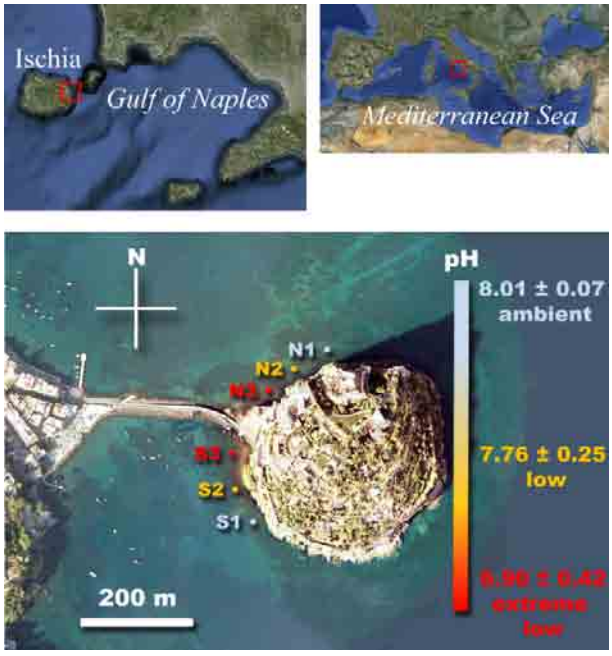

Figure 1. Map of the vent site at the Castello Aragonese (Ischia Island) with sampling stations and pH conditions.

Material and methods

Samples of the brown alga *Dictyota dichotoma* were collected on 5 dates in summer 2011 and 2012 at a depth of about 2 m. At each of the 6 stations, three macroalgal samples were collected by scuba diving at a distance of less than 10 m from each other. Macroalgal samples were gently put into 50 ml Falcon™ tubes during collection. Samples were fixed in laboratory with 0.4% neutralized formaldehyde and stored in the dark until the analyses. To assess *O.* cf. *ovata* concentrations, Falcon™ tubes were vigorously shaken to allow the detachment of *Ostreopsis* cells. The fresh weight of the macroalgal thalli (g fw) and the volume of the water sample containing detached cells inside the Falcon™ tube were measured (ml). Cells counting was performed using a Zeiss Axioskop 2 plus microscope at a magnification of 125x, on 1 ml of the water sample allowed to settle in a Sedgewick-Rafter slide (average detection limit: 360 cell g^{-1} fw). Counts were performed on 54 out of the 90 samples collected, as the remainder showed no or rare cells. Abundances of *Ostreopsis* cells were expressed as cells g^{-1} fw. Differences in *Ostreopsis* cell abundances within and between the two sites were evaluated through univariate and multivariate (main effects) analysis of variance (ANOVA) using Excel Stat software. P-value significance level was set at <0.05.

Results

Ostreopsis cells were rare on two of the five sampling dates (21 June 2011 and 27 July 2012). On the other dates, values ranged from 299.7 cells g^{-1} fw (2 July 2012) to $1.15 \cdot 10^6$ cells g^{-1} fw (21 July 2011). The highest values were recorded in July 2011 at all the northern, exposed stations ($7.8 \pm 2.4 \cdot 10^5$ cells g^{-1} fw, Fig. 2). On the same date, ca. ten-fold lower values ($7.4 \pm 3.9 \cdot 10^4$ cells g^{-1} fw), were recorded at the southern, sheltered stations. Differences between northern and southern stations were minor but still significant on 2 July 2012 (Table 1), when much lower values were recorded at both the northern ($8.5 \pm 5.8 \cdot 10^3$ cells g^{-1} fw) and southern stations ($2.0 \pm 1.3 \cdot 10^3$ cells g^{-1} fw). The third positive sampling on 13 July 2012 showed still lower values (avg. $8.1 \cdot 10^3$ cells g^{-1} fw), with no significant differences between north and south stations.

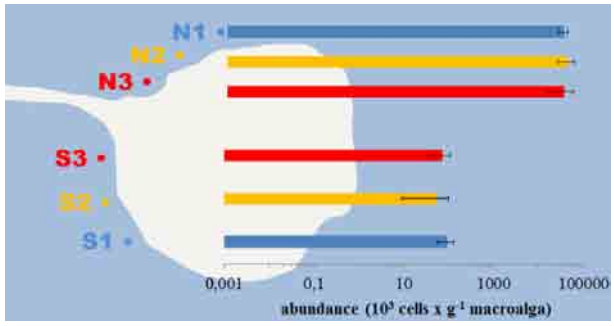

Figure 2. *Ostreopsis* cf. *ovata* abundance on 21 July 2011 at Castello Aragonese.

Coefficients of variation of abundances at the individual stations were high at both sides of Castello Aragonese in the same date (max.: 0.84, S2 station; 0.45, N3 station, 21 July 2011), ranging between 0.18 and 0.91 on the other dates. Within each site (northern and southern), in no case was the difference

significant among the stations of each sites. Multivariate ANOVA analysis confirmed the difference between the stations located in either sheltered or exposed sites and the lack of significant differences along the pH gradients.

Table 1. Univariate and multivariate ANOVA analysis on *O.* cf.. *ovata* abundance values within (pH gradient) and between (N/S) northern and southern stations. Significant variable effect (p-value) is marked in bold red.

	Univariate			Multivariate
	21 July 2011	2 July 2012	13 July 2012	
pH gradient	0.889786	0.054721	0.708202	0.231666
N/S	**0.000001**	**0.002213**	**0.945006**	**0.000006**

Discussion

Ostreopsis cf. *ovata* shows a marked seasonality, with peaks occurring during the early summer in the northwestern Mediterranean Sea (Mangialajo *et al.* 2011). Taking into account this temporal variability, the sampling was planned in June and July, when *Ostreopsis* abundances reach their maximum also at most sites of the Gulf of Naples (ARPA Campania, unpublished data). Accordingly, concentrations were high in July in both years, although in 2011 cell density was consistently higher than in 2012. Differences in abundance recorded at Castello Aragonese between the two years seem to be in agreement with what has been found in other Mediterranean sites, where *Ostreopsis* blooms show large inter-annual variability in relation to sea water temperatures and hydrodynamics (Asnaghi *et al.* 2012). It is also possible, however, that the bi-weekly scale of sampling missed the peak abundance in 2012. Abundance values attained at Castello Aragonese in 2011 were comparable to maximum epiphytic abundance values recorded in the northern Mediterranean Sea (Mangialajo *et al.* 2011), but higher than the North Aegean Sea ones (Aligizaki *et al.* 2008).

Differences in *O.* cf. *ovata* abundance between the northern and southern side of the bridge connecting the Castle to the Ischia Island were instead significantly different at least on two dates (Table 1). Further analysis on environmental conditions at the two sites are needed, but it is likely that physical drivers such as wave exposure and currents dynamic could play a synergistic effect favouring the attainment of high abundances in the exposed site as compared to the sheltered one. A preference for high energy waters has been reported for *Ostreopsis* cf. *siamensis* along the Spanish coasts (Vila *et al.* 2001), as well as for *Ostreopsis* sp. in coral reef habitats (Carlson and Tindall 1985).

Coastal waters at the two sides of the bridge may also differ in chemical factors, as the northern side could be more exposed to inputs from nutrient-rich filaments from a nearby river mouth (Iermano *et al*. 2012). Moreover, presumably lower irradiances in the northern, shadowed side of Castello Aragonese (Fig. 1) may favour more intense likely due to preference of *Ostreopsis* for shaded areas (Ballantine *et al.* 1988), and possibly photosaturation (Scalco *et al*. 2012) at low irradiances and photoinhibition at high light intensity values. To confirm these hypotheses chemical and physical conditions at the two sites should be investigated in further studies.

Spatial heterogeneity at small spatial scales is well known for *Ostreopsis* cf. *ovata* (Cohu *et al.* 2009) and it has been recorded also in our study, as highlighted by relatively high coefficients of variation among the three replicates at each station within a same date. Nonetheless, no significant differences in the abundance values were recorded among the three sites at each side of the bridge, despite the marked differences in pH values. These results show no effect of low pH condition on the spatial distribution of *O.* cf. *ovata*, indicating that this species can occur in the natural environment, and also reach bloom concentrations, at pH values definitely lower than the predicted value for 2100.

This result diverges from those obtained for other epiphytic organisms at the same vent site. Significant reduction was shown in coralline algal cover (Martin *et al.* 2008) and epiphytic diatoms assemblages (Porzio *et al.* 2012), involving qualitative and quantitative changes in community composition. Variations along the pH gradient were also shown for invertebrate communities (Kroeker *et al.* 2011). These spatial differences in other biological compartments, however, do not seem to negatively affect the intensity of *Ostreopsis* blooms along the pH gradient. In contrast, the absence of sea urchins in the low pH sites (Hall-Spencer *et al.* 2008) causes an alteration in macroalgal community with a prevalence of fleshy macroalgae such as *Dictyota dichotoma* (Porzio *et al.* 2011), which are more suitable hosts for *Ostreopsis*. This situation presents an analogy with the case of

tropical areas, where the demise of coral reefs, as a consequence of ocean acidification and increased temperature, can lead to an increase of macroalgae, more prone to colonization by harmful benthic dinoflagellates (Hallegraeff 2010).

Conclusions

Naturally acidified areas are useful laboratories to test feedbacks and indirect effects occurring among interacting organisms within natural marine systems, otherwise difficult to replicate in mesocosm and laboratory conditions (Thomsen *et al.* 2010). The present study provides the first quantitative description of an intense *Ostreopsis* bloom in a natural acidified area. *Ostreopsis* cf. *ovata* appears to be tolerant to a wide range of pH values. Therefore, ocean acidification is not likely to represent a hindrance to the spreading and intensification of its blooms. Differences observed between sheltered and exposed sites rather highlight the rôle of hydrographic factors, either directly or through their effects on the biota in the two sites. Changes in these factors under the pressure of climate change could be major drivers for long term trends of *Ostreopsis* blooms.

Acknowledgements

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Exploring the conditions leading to an exceptional early bloom of *Dinophysis acuminata* in northwest Spain during 2012

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Abstract

In a conceptual model proposed before, the onset of the upwelling season and availability of *Mesodinium rubrum* prey are essential conditions for the initiation of *Dinophysis acuminata* blooms in the Galician Rías Baixas (NW Spain). Distribution of sexual cysts from the previous year in the top sediment layer is an important parameter in forecasting models of cyst-forming species (e.g. *Alexandrium* spp.). But the existence of sexual cysts of *Dinophysis* spp. has not been confirmed, *Dinophysis* planozygotes can divide and produce vegetative cells directly, and it is possible that species of this genus do not rely on sexual cysts but on some kind of overwintering planktonic form as a seeding mechanism. Here we examine winter conditions—persistence or absence of overwintering cells of *Dinophysis* within the rías and the adjacent shelf and time of initiation of the upwelling season—from observations in the last 20 years. Our ultimate goal was to explain the abundance and early initiation in April 2012 of an extraordinary bloom of *D. acuminata*, in what appeared to be a mesoscale event affecting other regions in Western Europe. The dynamics of *D. acuminata* populations appeared tightly coupled to the upwelling season. Our results suggest that anomalous upwelling patterns (predominance of upwelling in winter) combined with the presence of overwintering (inoculum) cells lead to early initiation of the *Dinophysis acuminata* growth season.

Keywords: Dinophysis acuminata, upwelling patterns, interannual variability, exceptional bloom, Galician Rías.

Introduction

Several species of *Dinophysis* produce lipophilic shellfish toxins (OA, DTXs, PTXs) and are the main cause of shellfish harvesting closures in Western Europe, becoming a chronic problem on the Atlantic coasts of Iberia, Brittany (France), SW Ireland, Skagerrak-Kattegat, and Norwegian Sea. *Dinophysis acuta* and in particular *D. acuminata* pose the main threat to the sustainable exploitation of blue mussels in Galicia (NW Spain), site of a production up to 300,000 t y^{-1}. *D. acuminata* populations may occur through the whole upwelling season (spring to early autumn), the first maxima (>10^3 cells L^{-1}) in early June (Velo-Suarez et al. *in press*). Thanks to monitoring inputs and research projects, considerable knowledge has been gained concerning seasonality and short-term response of *Dinophysis* spp. to environmental conditions. But we still know very little about the causes of their inter-annual variability. Single-species predictive models require a sound knowledge of the biology of the target organism. In cyst-forming dinoflagellates, sexual cysts density on the top sediment layer is a very important parameter, the potential inoculum, used in forecast models (Anderson et al., 2005). Observations of planozygote division—with no need of sexual cyst maturation—and the rare occurrence of putative cysts (sometimes misidentified *Fragilidium* —that recently fed on *Dinophysis*—asexual cysts) during exceptional blooms support the view that *Dinophysis* spp do not rely on resting cysts germination as a seeding strategy (Escalera and Reguera, 2008). Identification of the "pelagic seed bed" (*sensu* Smayda, 2002) and the way it is introduced in the Galician Rías. This paper examines the winter conditions in the last 20 years to explain the exceptional early bloom of *D. acuminata* in 2012.

Material and Methods

The Galician Rias Baixas (NW Spain) (Fig. 1) are located in the northern limit of the Canary Current upwelling system. This region is under the influence of the North Atlantic weather system, where the meteorological dynamics is highly conditioned by the seasonal evolution of two atmospheric systems, the Azores high-pressure and the Iceland low-pressure (Wooster *et al.* 1976). The displacement of atmospheric high-low pressures, favouring upwelling or downwelling events determines a seasonal upwelling regime, from April to October (McClain *et al.* 1986).

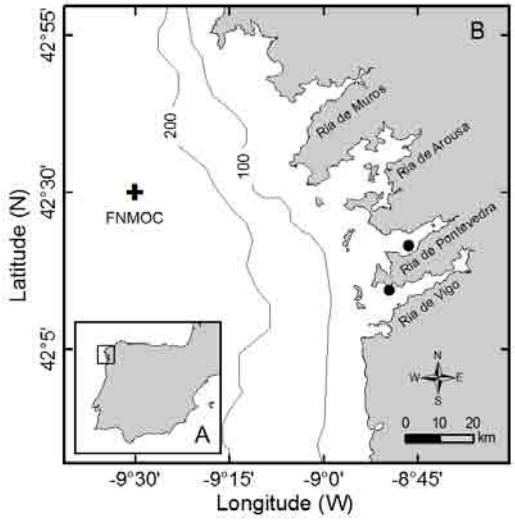

Fig. 1. Galician Rías Baixas (NW Spain), showing the location of the 2 "*hot spots*" (black circles) and the position for the Ekman transport estimates (black cross).

Time series of days of harvesting closures at the "hot spots" in Ria de Vigo and Ria de Pontevedra and weekly reports on phytoplankton distribution at the Ria de Pontevedra from 1993 to 2012 were obtained from the Galician Monitoring Program (INTECMAR). Monthly reports of phytoplankton distributions in a transect from Ria de Vigo to the adjacent shelf were obtained from the programme *Radiales* (IEO). The time series of six-hourly Ekman transport data over the last 46 years (1967–2011), were estimated using model data from the U.S. Navy's Fleet Numerical Meteorology and Oceanography Centre (FNMOC) derived from Sea Level Pressure (www.indicedeafloramiento.ieo.es) on a grid of approximately 1°x1° centred at 42.5°N 9.5°W, representative for the study area (Fig. 1).

Results and discussion

The days of harvesting closures per year at the hot spots in the Ria de Vigo and Ria de Pontevedra evidenced a high interannual variability, with minimum days of closure in 1996 and 1997 and maxima on 1995, 2004 and 2005 (Fig. 2).

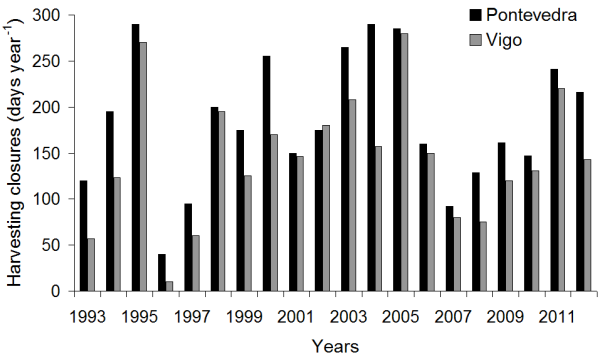

Fig. 2. Days of harvesting closures per year (lipophilic toxins) at the hot spots in Ria de Vigo and Ria de Pontevedra.

The monthly average Ekman transport ($m^3\ s^{-1}\ km^{-1}$) showed a typical pattern described by McClain et al. (1986) and characterized by an upwelling-season from April to October with winter and autumn transitions in March and October (Fig. 3).

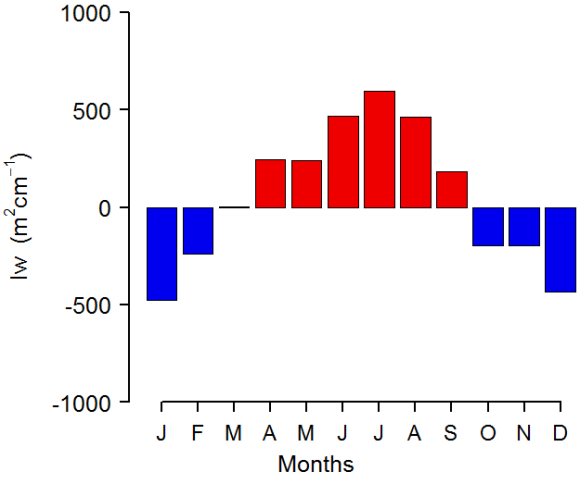

Fig. 3. Monthly averaged (1967-2011) Ekman transport ($m^3\ s^{-1}\ km^{-1}$) estimated from the FNMOC-model.

The inter-annual variability of *D. acuminata* during the last 20 years showed maximum cell concentrations in 2010-2012, and minimal densities in 1996 and 1997 (Fig. 4).

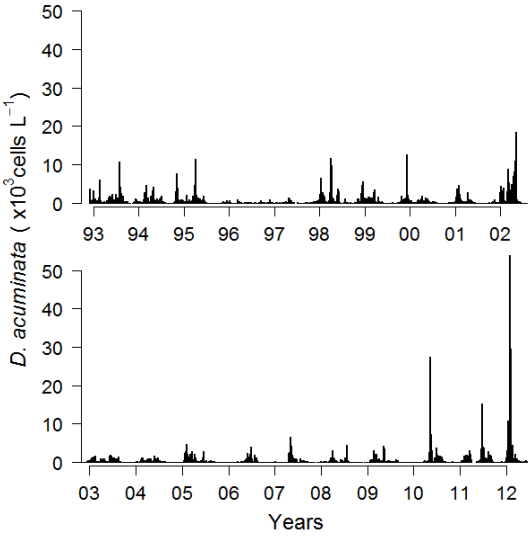

Fig. 4. Inter-annual variability of *D. acuminata* from 1993 to 2012 in the Ria de Pontevedra.

During 2012 an extraordinary bloom of *D. acuminata* was observed at the Rias Baixas in terms of abundance and early initiation. A maximum of $6x10^4$ cells L^{-1} was observed in integrated hose samples (0-5m) at a shallow station in the innermost part of Ria de Pontevedra in April (Fig. 5).

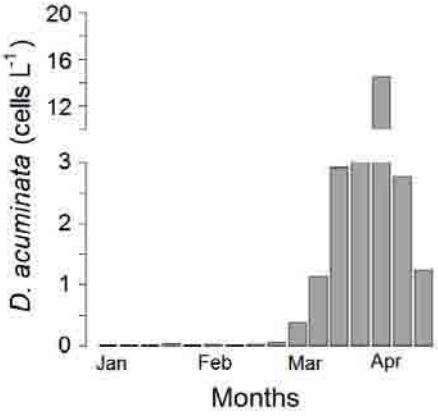

Fig. 5. Weekly average of *D. acuminata* densities ($x10^3$ cells L^{-1}) from 10 stations (integrated hose samples) in Ria de Pontevedra from January to April 2012.

The analysis of the time-series of *D. acuminata* distribution evidenced that years with early bloom initiation (April)—e.g. 2000, 2005 and 2012—coincided with anomalous patterns of Ekman transport characterized by upwelling-dominance during the winter months (Figs. 6, 7). In contrast, years characterized by late initiation of *D. acuminata* blooms (July – September)—mainly 2002 and 2010—coincided with normal upwelling patterns, i.e. dominance of downwelling in the winter months (Figs. 6, 7).

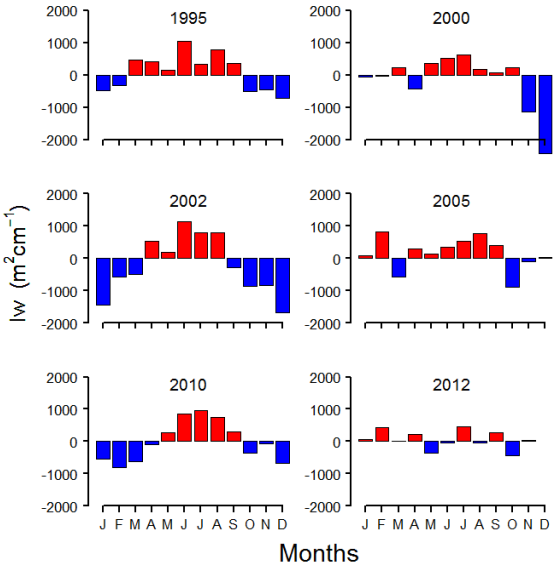

Fig. 6. Ekman transport ($m^3s^{-1}km^{-1}$) during selected years with normal (left) and anomalous (right) upwelling patterns in relation to the historical monthly mean.

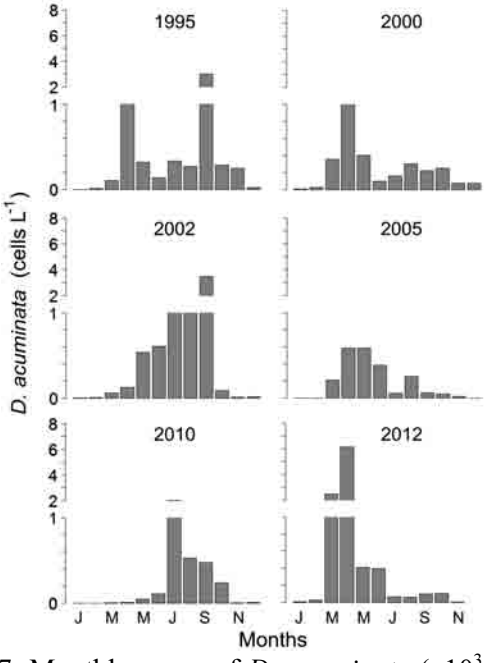

Fig. 7. Monthly mean of *D. acuminata* ($x10^3$ cells L^{-1}) in Ria de Pontevedra during selected years with normal (left) and anomalous (righ) upwelling patterns.

The presence of scattered overwintering cells of *Dinophysis* (Jan-Feb) that would act as the inoculum population is tested here as one of the key factors explaining early initiation of the blooms. This mechanism is the essence of the "pelagic seed bank" conceptual model proposed by Smayda (2002). This model proposes a pelagic analogue of the "seed (cysts) beds" deposited onto the sea sediments, in particular for species that do not rely on sexual cyst germination as their inoculum. In this context, years of early high densities of *D. acuminata* in the Ria de Pontevedra during the last 20 years—mainly 2000 and 2012—appeared associated with the presence of overwintering cells in combination with anomalous winter upwelling patterns (Table 1). That was the case in Ria de Pontevedra in 2012, when the presence of scattered individual cells during the winter months was evident (Fig. 8).

Table 1. Maximum cells concentration of *D. acuminata* during winter months in Ria de Pontevedra from 1993 to 2012.

Year	Months											
	January				February				March			
1993												
1994												
1995												
1996												
1997												
1998												
1999												
2000												
2001												
2002												
2003												
2004												
2005												
2006												
2007												
2008												
2009												
2010												
2011												
2012												
Cells L^{-1}	0		100		1000		10000		>10000			

Conclusions

The initiation of the growth season of *D. acuminata* appears strongly coupled to the upwelling season. Early initiation of the blooms seems also associated with the presence of overwintering cells in the outer reaches of the Rías and their adjacent shelf. A combination of these two factors was associated to the exceptional intense bloom of *D. acuminate* in 2012.

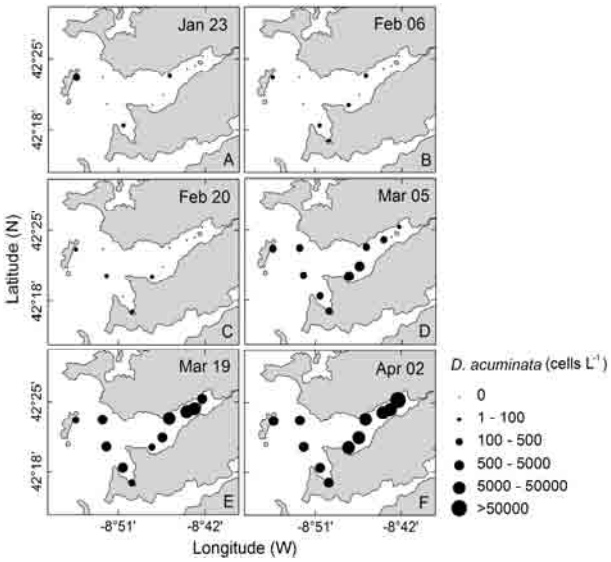

Fig. 8. Spatial and temporal distribution of *D. acuminata* at the Ría de Pontevedra from January 23 to April 02, 2012.

Acknowledgements

We thank INTECMAR for weekly reports on phytoplankton distributions and shellfish harvesting closures in the Galician Rías and IEO programme RADIALES for monthly reports on phytoplankton distributions. This work was funded by project ASIMUTH (EU 7FM grant # 261860) and by a PhD grant to Patricio A. Díaz from CONICYT (Chile). This is a contribution to the SCOR and IOC programme on "*Global Ecology and Oceanography of Harmful Algal Blooms* (GEOHAB)", Core Research Projects on "*HABs and Stratification*" and "*HABs in Upwelling Systems*".

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Velo-Suárez, L. González-Gil, S., Pazos, Y., et al. (2014). Deep-Sea Res. II. 101: 141-151.

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Spring-neap tidal and circadian variability in the distribution of two groups of *Pseudo-nitzschia* species in an upwelling-influenced estuary

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Abstract

High-resolution physical and biological measurements were carried out in the Ría de Pontevedra (NW Spain) in late spring during the 'HABIT Pontevedra 2007' survey, which utilized high vertical resolution instruments. Cell maxima of *P. delicatissima* (6 x 10^5 cells L^{-1}) and *P. seriata* (2 x 10^6 cells L^{-1}) groups were observed during the first half of the cruise during downwelling and a significant decrease in cell numbers occurred during subsequent upwelling conditions. The effect of tidal (both semidiurnal and spring-neap) and event driven (upwelling-downwelling cycle) variability were evident. The observed sequence of events suggests that *Pseudo-nitzschia* populations were advected from the shelf. The circadian variability was regulated by tidal forcing and *Pseudo-nitzschia* spp. maxima were observed at low tide. From results presented here we conclude that the magnitude of spring-neap tidal and circadian variability has to be considered when designing and implementing harmful algal bloom monitoring programmes.

Keywords: *Pseudo-nitzschia* spp; upwelling-downwelling cycle, spring-neap tidal variability, circadian variability, Galician Rías.

Introduction

Pseudo-nitzschia spp. of the *seriata* and *pseudo-delicatissima* groups are associated with chronic ASP events in the Galician Rías Baixas (Fraga *et al.* 1998). Toxic outbreaks are short-lasting in mussels but toxins are accumulated for very long periods (months or even permanently) in scallops, a fact that led to exceptional procedures approved by the EU to regulate ASP toxins in pectinid bivalves (Salgado *et al*. 2003). The Galician Rías, are a site of intensive shellfish production—300 x 10^3 t y^{-1} of blue mussels and 17 x 10^3 t of other shellfish species per year (FAO, 2012) and there are therefore considerable socioeconomic impact of toxin producing microalgae in general and of *Pseudo-nitzschia* spp. This paper evaluates the effect of spring-neap tidal and circadian variability in the distribution of species of the *Pseudo-nitzschia delicatissima* and *P. seriata* groups, for blooms which developed during a downwelling-upwelling cycle.

Material and Methods

The study was carried out on board R/V *Mytilus* from 28 May to 8 June 2007 in Ria de Pontevedra (Fig. 1). Measurements of physical properties of the water column were carried out with an IPSAP (IFREMER Particle Size Analyzer Profiler) probe, a Scanfish-PS19 towed fish, and profiles of current velocity with a moored ADCP (Acoustic Doppler Current Profiler). Water samples were collected for nutrient analyses and for quantitative analyses of phytoplankton to describe the spring-neap and circadian tidal variability of *Pseudo-nitzschia* spp. *Pseudo-nitzschia* species were separated in two groups, *P. delicatissima* and *P. seriata*, depending on their valve width (Hasle 1965).

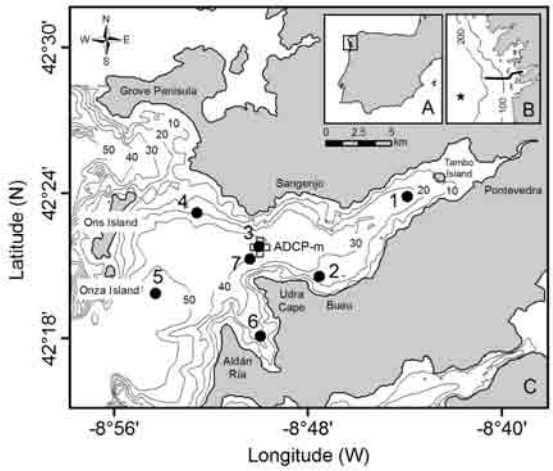

Fig. 1. Ría de Pontevedra (Galician Rías Baixas, NW Spain), showing the location of the 7 sampling stations and the ADCP-mooring site.

Results and discussion

Southerly winds during the first half of the cruise, generated onshore Ekman transport (620 m^3 s^{-1} km^{-1}), whereas the onset of northerly winds during the second half led to a reversed circulation, with a maximum estimate of 1535 m^3 s^{-1} km^{-1} (offshore Ekman transport) (Fig. 2).

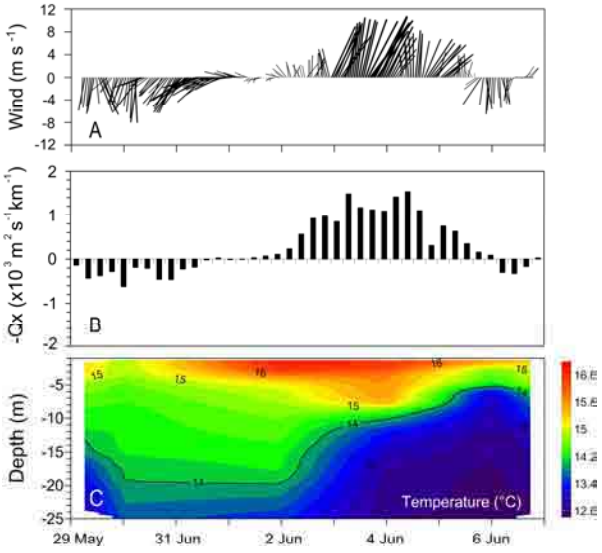

Fig. 2. Changes of A) wind direction and velocity B) Ekman transport and C) temperature from 29 May to 7 June 2007 at station 2.

The survey coincided with the annual onset of *Pseudo-nitzschia* blooms in the Galician Rías Baixas. The *P. seriata* group was more abundant than that of *P. delicatissima* throughout the cruise. The spatial and temporal distribution of the *P. seriata* group showed higher cell densities in the first half of the survey with a maximum of 2 x 10^6 cells L^{-1} (Fig. 3). *P. delicatissima* group showed a similar pattern to that of the *P. seriata* group but with lower cell densities and a maximum of 6.6 x 10^5 cells L^{-1} (Fig. 4).

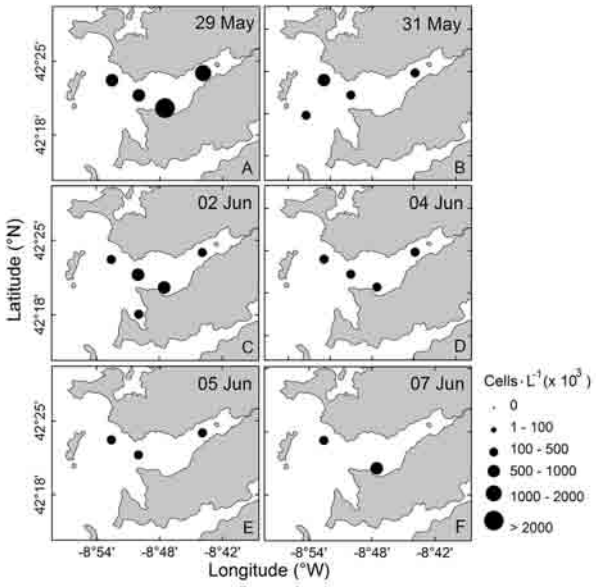

Fig. 3. Spatial and temporal evolution of *P. seriata* (x 10^3 cells L^{-1}) during the survey.

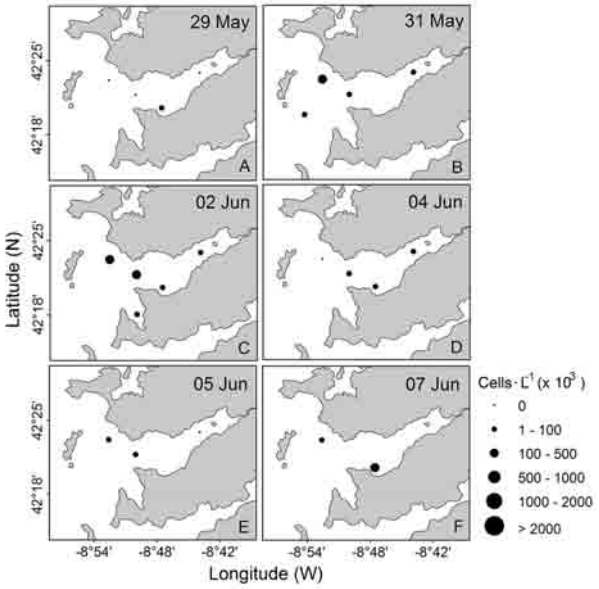

Fig. 4. Spatial and temporal evolution of *P. delicatissima* (x 10^3 cells L^{-1}) during the survey.

Circadian variability of the *P. delicatissima* and *P. seriata* groups during the two 24-h cycles at station 2 seemed to be dominated by tidal forcing. During both 24-h cycles, the *P. seriata* group was dominant. Intratidal distribution showed cell maxima of *P. delicatissima* and *P. seriata* groups appeared at low tide during maximum stratification (Figs. 5 and 6).

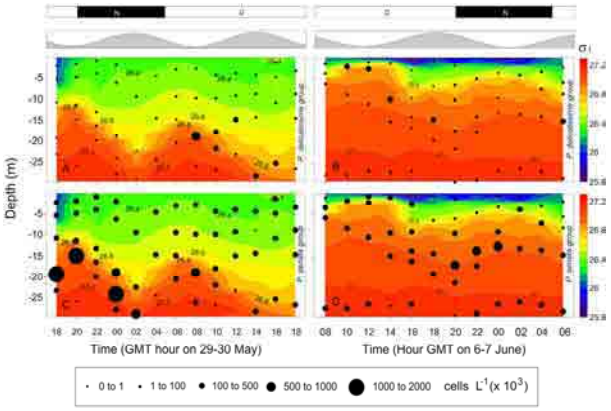

Fig. 5. Intratidal distribution of *Pseudo-nitzschia delicatissima* and *P. seriata* groups (x10^3 cells L^{-1}) sampled during two 24h-experiments at station 2.

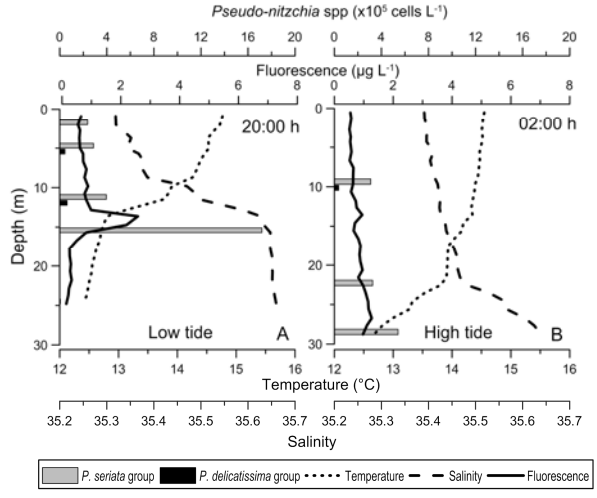

Fig. 6. Vertical distribution of *P. delicatissima* and *P. seriata* groups and CTD-profile during low (A) and high tide (B).

Results here show a different mechanism of bloom formation than that described by Velo-Suárez *et al.* (2008) in the same location. These authors showed maximum densities of *Pseudo-nitzschia* spp (>1.5x10^6 cells L^{-1}) forming thin layers located within a very steep pycnocline (~2 kg m^{-3} /10 m) under upwelling-favourable winds; sinking and erosion of the thin layer—that included a senescent population of *Pseudo-nitzshia* spp.—was then associated with downwelling-favourable winds.

The spring-neap tidal variability of *Pseudo-nitzschia* groups was observed also from MERIS and MODIS satellite images (ground-truthed with plankton counts), which recorded a top water layer displacement of high concentrations of chlorophyll towards the coast on 28 May (Fig. 7).

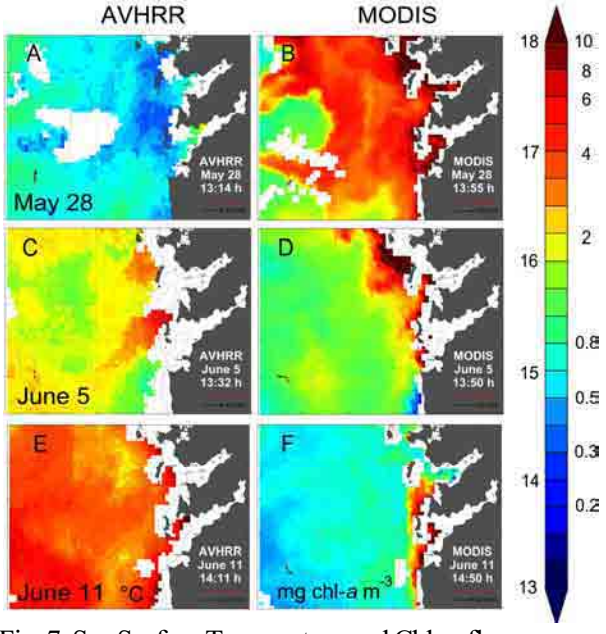

Fig. 7. Sea Surface Temperature and Chl-*a* fluorescence estimates from AVHRR and MERIS satellite data during the cruise period. White patches represent clouds.

This kind of filaments have been reported to constitute an important mechanism of cross-shelf transport of phytoplankton populations during upwelling events in the Iberian upwelling system (Barton *et al.* 2001, Smyth *et al.* 2001) and may be responsible for the significant decrease in *Pseudo-nitzschia* densities observed during the second half of the cruise.

Conclusions

Hydrodynamic control is crucial in the formation, transport and dissipation of *Pseudo-nitzschia* spp. blooms in the Galician Rías Baixas (NW Spain). Upwelling-downwelling cycles, modulated by semidiurnal and spring-neap tides are the main forcing elements controlling the formation, transport and dissipation of *Pseudo-nitzschia* populations.

The sudden increase of *Pseudo-nitzschia* densities

were caused by physical advection, in contrast with previously described scenarios of *in situ* growth and thin layer formation in the Galician Rías. *Pseudo-nitzschia* blooms were tracked with MERIS and MODIS satellite images, which recorded a surface displacement (approx. 0-10 m) of high concentrations of chlorophyll towards the coast.

Acknowledgements

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Alexandrium catenella cyst distribution and germination in Puget Sound, WA USA

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Abstract

The PS-AHAB (Puget Sound *Alexandrium* Harmful Algal Bloom) program, funded by NOAA/ECOHAB, seeks to understand environmental controls on the benthic (cyst) and planktonic life stages of the toxic dinoflagellate *Alexandrium catenella*, and evaluate the effects of climate change on the timing and location of blooms. This includes detailed mapping of overwintering cysts at 99 stations throughout Puget Sound. Highest surface sediment cyst abundances in 2011 and 2012 were found in Bellingham Bay (north), in bays on the western side of the central main basin and in Quartermaster Harbor (south). While cyst distribution patterns were similar for both years, 2012 cyst abundances were a factor of two lower at most stations. Compared to a 2005 survey, the Bellingham Bay "seed bed" is new, whereas Quartermaster Harbor cyst concentrations have decreased by an order of magnitude. In a related study funded by Washington Sea Grant, cysts from surface sediments at thirty 2012 PS-AHAB stations were evaluated for their germination potential with results ranging from 16% to 66% viability. To date, no relationship between cyst viability and cyst appearance has been detected. These results will be used to inform a model to explore the possibility of providing seasonal *Alexandrium catenella* bloom forecasts.

Keywords: *Alexandrium catenella*, cysts, sediments, germination, Puget Sound

Introduction

Paralytic shellfish toxins (PSTs) have been present in the Puget Sound region for centuries (Quayle 1969), however, little is known about the local distribution or biology of the causative organism *Alexandrium catenella*. *A. catenella* is a dinoflagellate that spends part of its life cycle as a cyst in the sediment before germinating to become a vegetative cell (Anderson 1998). This species produces a suite of neurotoxins (PSTs), the most potent being saxitoxin (Anderson *et al.* 1990). PSTs can accumulate in the tissues of filter-feeding shellfish, and be lethal in small doses to humans if consumed. *A. catenella* blooms therefore pose significant problems for local human health officials, marine resource managers and shellfish growers.

The Puget Sound *Alexandrium* Harmful Algal Bloom PS-AHAB study is a three year project (http://www.tiny.cc/psahab) funded by NOAA/ ECOHAB designed to 1) map interannual variations in *A. catenella* cyst distribution in Puget Sound, 2) do laboratory experiments to quantify the rates and timing of cyst germination related to exogenous and endogenous factors, 3) integrate the results from the first two objectives into coupled hydrodynamic and climate models to determine current favorable habitat areas for *A. catenella* and evaluate the effects of climate change on these habitats in the future, and 4) establish a time series with sufficient depth to provide seasonal forecasts of harmful algal blooms. In a related study, funded by NOAA Seagrant, we are investigating how cyst distribution in surface sediment changes over the course of a year in one bay. We are also running experiments to determine mandatory dormancy, secondary dormancy and cyst viability for *A. catenella* in Puget Sound. Results from the two years' surveys (2011 and 2012) of *A. catenella* cyst distribution in the surface sediments of Puget Sound are presented here and compared to an earlier surface sediment cyst survey from 2005 (Horner *et al.* 2011). Cyst germination viability experiment results from selected 2012 survey samples are also reported here.

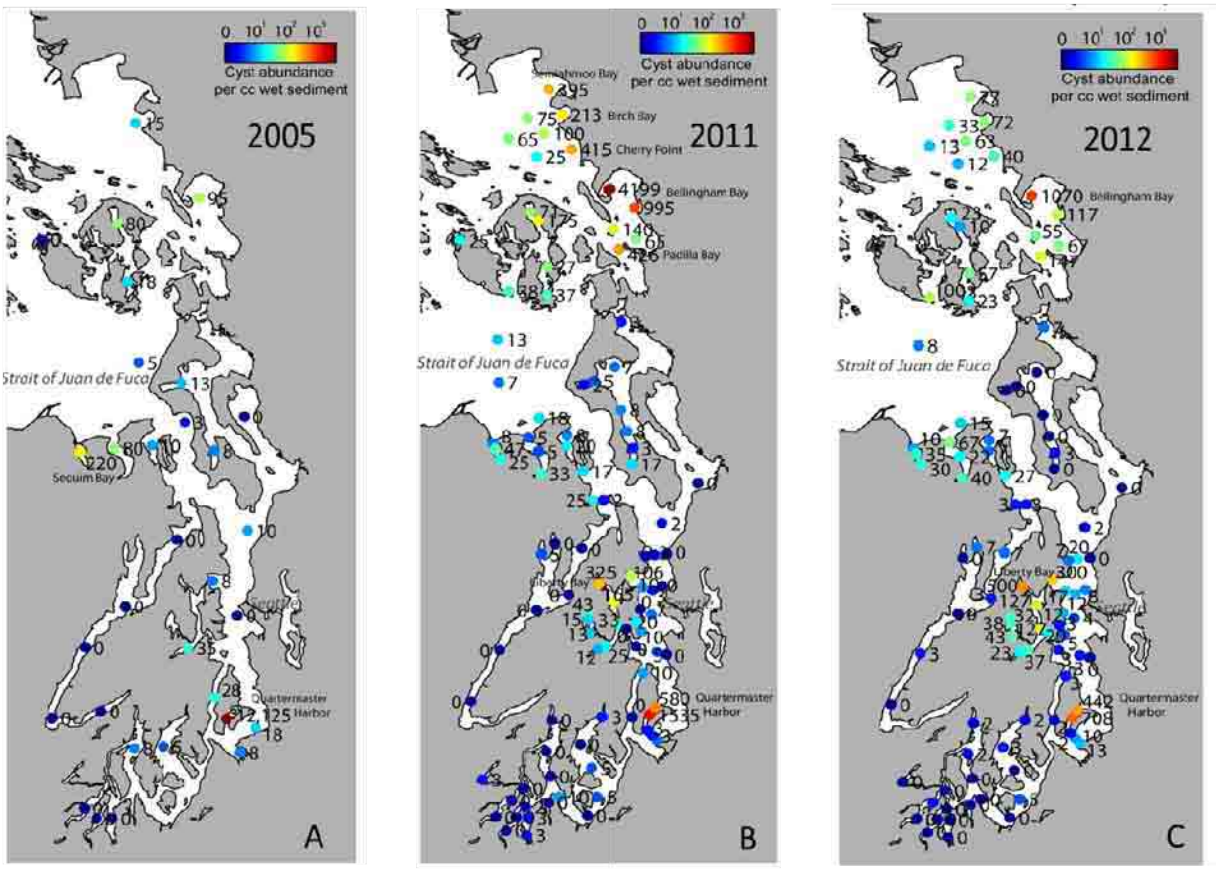

Fig. 1. Puget Sound surface sediment cyst abundances per cubic centimeter wet sediment. All surveys were done in the winter of each year. A) 2005; B) 2011 and C) 2012

Material and Methods

Field Sampling & Cyst Enumeration: Surface sediment *A. catenella* cyst distribution mapping surveys were completed during winter in 2011 and 2012. Surveys consisted of 99 stations throughout all of Puget Sound, the Strait of Juan de Fuca and the San Juan Islands (Fig. 1). Sediment samples from the upper 0-1 cm and 1-3 cm were collected using a Craib corer. (Anderson *et al.* 2003). Sediments were processed for cyst enumeration using the method of Yamaguchi *et al.* (1995), total organic content (loss-on-ignition) and grain size using a particle size analyzer.

Cyst Viability: Additional surface (0-1 cm) sediment samples were collected from 30 stations throughout Puget Sound during the 2012 winter survey using the Craib corer. Stations were chosen where there was greater than 25 cysts/cc sediment from the 2011 survey. Samples were stored at 4°C, in the dark, in nitrogen gas bags until cyst isolation could be performed (10-46 weeks after collection). One cubic centimeter of sediment was diluted to 50 ml with filtered seawater, sonicated and sieved through 90 um and 20 um sieves with the 20-90 um size fraction retained. After settling, sub-samples of this size fraction were placed on a Sedgewick-Rafter slide and individual cysts were picked using a micropipette. The first 60 cysts of *Alexandrium* encountered while scanning through the Sedgwick-Rafter slide were isolated from the 20-90 um size fraction of sediment from each of the 30 stations. Picked cysts were placed in a Palmer-Maloney slide for holding and as a rinsing step, and then one cyst per well was placed into a 96 well plate rack with 200 uL nutrient enriched natural seawater growth media. Well plates were incubated at 4°C with a 14:10 light:dark cycle. Light levels were 70-90 uEM-2s-1 based on in situ data collected at the bottom of Quartermaster Harbor. Each plate was photographed and checked for germinated swimming cells at days 5, 14 and 28 and the number germinated recorded for the first 50 wells. Replicate isolations were conducted using sediment from the Quartermaster Harbor and Bellingham

Bay stations at 10 and 20 weeks after collection to test for any effects of storage time on cyst viability.

Results

Highest cyst concentrations were found in Bellingham Bay, Birch Bay and Semiahmoo Bay in the north, Port Madison, Liberty Bay and Port Orchard on the west side of the main basin and Quartermaster Harbor in central Puget Sound (Fig. 1). Quartermaster Harbor 2011 cyst concentrations were an order of magnitude less compared to a 2005 survey (Horner *et al*. 2011), and a new "seed bed" area has developed in Bellingham Bay. 2012 cyst distribution patterns are the same as 2011, but cyst concentrations are generally less in the surface sediment layer. Cyst germination viability ranged between 16-66% with no apparent difference between the surface (0-1cm) and 1-3cm layers (Table 1). To date, no relationship between cyst viability and cyst appearance using image analysis software has been detected.

Table 1. 2012 Puget Sound surface sediment cyst abundances and cyst germination viability.

#	Station	2012 cysts/cc in sediment	Germination
1	Semiahmoo Bay	77	37%
4	Birch Bay	72	34%
8	Bellingham Bay - North (0-1cm)	1070	48%
8	Bellingham Bay - North (0-1cm)	1070	54%
8	Bellingham Bay - North (1-3cm)	3830	44%
9	Bellingham Bay - East	117	52%
10	Bellingham Bay - South	67	44%
11	Bellingham Bay - West	55	48%
12	Padilla Bay	147	30%
15	Lopez Sound - Outer	57	20%
17	Cattle Point (Van Veen)	100	32%
22	Sequim Bay - Center	35	34%
58	Port Madison	300	42%
59	Liberty Bay	500	36%
60	Port Orchard - North	127	46%
61	Port Orchard - South	127	54%
78	Quartermaster Harbor - Center (0-1cm)	708	16%
78	Quartermaster Harbor - Center (0-1cm)	708	38%
78	Quartermaster Harbor - Center (1-3cm)	1022	66%
79	Quartermaster Harbor - Inner	442	42%

Discussion and Future Work

The preliminary maps from the annual cyst distribution surveys are shared with Puget Sound health officials, marine resource managers and shellfish growers in the spring of each year, as part of the PS-AHAB "just-in-time" information dissemination to stakeholders program. One more year of field samples will be collected and analyzed to determine the interannual variability of *A. catenella* cyst distribution in the surface sediments of Puget Sound and provide shellfish growers with an early warning map of potential high bloom areas. Laboratory experiments investigating the rates and timing of cyst germination related to exogenous and endogenous factors are ongoing. In a related study, we are also doing mandatory and secondary dormancy experiments, as well as continuing the viability study. In addition, we are investigating the variation in surface sediment cyst distribution in Quartermaster Harbor monthly for one year. These data will then be used to inform coupled Puget Sound hydrodynamic and climate models to explore the possibility of providing seasonal harmful algal bloom forecasts in the future.

Acknowledgements

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The relationship between harmful algal blooms and cyst germination in Korean waters

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Abstract

It has been known that the germination of resting cysts plays a key role in the onset of algal blooms in previous studies. The population dynamics of *Heterosigma akashiwo*, *Peridinium claudicans*, and *P. conicum*, a multi-modal blooming species, support this hypothesis. In a laboratory experiment, the rejuvenation of *H. akashiwo* resting cells, which was induced by exposure to darkness, low temperature, and limited nutrients, occurs without a dormant period. This may explain why the population dynamics of this species in the field seem to be characterized by sporadic appearance and rapid disappearance of blooms. On the other hand, *Scrippsiella trochoidea*, *Alexandrium tamarense*, and Nostocales, including *Anabaena cylindrica,* had a temporal discrepancy between the peak of the vegetative population and the germination rate. The reason for the delay from germination to algal bloom remains unclear. This paper reviews the role of resting cells in the initiation of algal blooms and suggests a new perspective on the relationship between harmful algal blooms and cyst germination.

Keywords: Harmful algal bloom, resting cells, germination.

Introduction

Many phytoplankton transform from a vegetative state into a resting cell, temporary cyst, resting cyst, or statospore (Imai and Itakura 1999; Kim *et al*. 2002), which permits over-wintering, survival from nutrient stress, and relocation to new regions through transport by way of natural currents or ocean vessels (Hallegraef and Bolch 1991). Furthermore, resting cells play a significant role in population dynamics. After the dormant stage, environmental signals trigger algal cysts to germinate into vegetative cells when growth conditions become more favorable (Corliss and Esser 1974). Thus, resting cyst germination directly leads to vegetative blooms (Anderson and Morel 1979; Imai and Itakura 1999).

However, not all studies support this scenario. For example, bloom initiation by *Scrippsiella trochoidea* did not always occur due to mass cyst germination (Ishikawa and Taniguchi 1994, 1996). Instead, algal blooms of this species appear to result from the dramatic growth of a small number of vegetative cells, and cysts may germinate after a vegetative bloom, rather than before. Studies of other dinoflagellates have also documented a significant delay between germination and vegetative blooms (Kim and Han 2000; Kim *et al*. 2002). Thus, depending on the species, cyst germination appears to have different roles in the triggering of algal blooms.

This study provides an overview of the role of resting cysts in the generation of algal blooms, and suggests a new perspective on the relationship between harmful algal blooms and cyst germination.

The different roles in the cyst germination mode of mono- and multi-modal blooming species

HABs (Harmful Algal Blooming species) can be characterized as mono-modal, having one germination and bloom peak each year, or as multi-modal, having several maxima of germination and blooming throughout the year. Based on the bloom dynamics, Heterosigma *akashiwo* (Han 1988), *P*eridinium *cunningtonii*, and *P. claudicans* (Ishikawa and Taniguchi 1997) belong to multi-modal blooming species, and *S. trochoidea*, Alexandrium *tamarense*, and Anabaena *cylindrica* are mono-modal blooming species. Interestingly, cyst germination plays an important role as a direct trigger of bloom initiation in multi-modal blooming species (Ishikawa and Taniguchi 1997). However, cyst germination of mono-modal blooming species is not always a major trigger for bloom initiation.

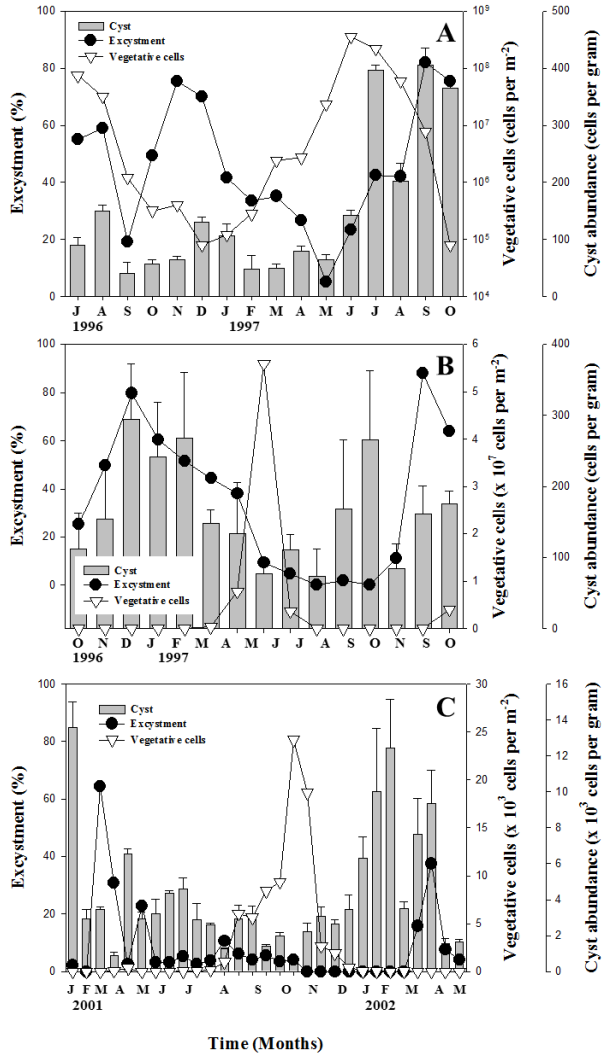

Fig. 1. Seasonal changes in cyst abundance (bar), excystment (circle), and vegetative cell density (triangle). A: *S. trochoidea* (Kim and Han 2000). B: *A. tamarense* (Kim et al. 2002). C: *A. cylindrical* (Kim *et al*. 2005).

In our results, *S. trochoidea*, *A. tamarense*, and *A. cylindrica*, regarded as mono-modal blooming species, have a time discrepancy between the maximum excystment and vegetative cell abundance (Fig. 1). Blooms of *H. akashiwo*, regarded as multi-modal blooming species, can occur and vanish at intervals of a few days (Han 1988). How can we explain the unique population dynamic of *H. akashiwo*? In general, algal vegetative cells have diverse alternative life cycles, such as asexual cysts (pellicle cysts). Sometimes, planozygotes can divide during the motile stage without encystment (Walker 1984). It is also known that the zygote develops into a unicellular cyst by sexual reproduction. However, in our study, none of the *H. akashiwo* resting cells transformed into resting cysts, and resting cells might have germinated without the mandatory period of dormancy (Fig. 2). This result suggested that *H. akashiwo* resting cells could not enter a quiescent state, remaining viable in the sediment. If so, this would allow for immediate germination when environmental conditions become more favorable for vegetative growth. Accordingly, we suggest that *H. akashiwo* produces propagules by two ways of life cycles Fig. 3). The one way is that resting cells transformed into natural cyst requiring dormant period prior to germinate. The other way is that resting cells germinate into vegetative cell immediately when condition changes favorable. Therefore, this combined "strategy" enables short-term sporadic and frequent blooms of H. akashiwo. However, to establish this scenario, efforts for finding the resting cells will need to be undertaken in the future, because resting cells of *H. akashiwo* have not yet been found in sediment from natural environments.

Why is the cyst germination of mono-modal blooming species not able to be a key trigger for the initiation of vegetative blooms? One possible explanation is that the bloom of these species may be limited by vegetative growth, rather than germination. Generally, vegetative cell growth in the water column is controlled by complex interactions among physical (temperature, light intensity, salinity), chemical (concentrations of nutrients), and biological factors (competition with other phytoplankton, grazing by zooplankton, algicidal effects of parasites, bacteria, viruses, *etc.*). In fact, we thought it would be difficult for mono-modal blooming species to synchronize the time between cyst germination and the proliferation of vegetative cells. Therefore, cysts of mono-modal blooming species need to have different germination strategies from that of multi-modal blooming species.

Anglès *et al*. (2012) reported that Ishikawa and Taniguchi (1996, 1997) identified three basic excystment patterns: *(i)* sporadic, in which there is irregular excystment throughout the year; *(ii)* synchronous, in which germination only occurs during a specific season; and *(iii)* incessant, in which there is continuous germination with marked peaks during the year. Incessant cyst germination can occur under conditions that may be unfavorable for vegetative cell growth, such as during the winter, and enables the immediate formation of blooms under favorable growth conditions, regardless of

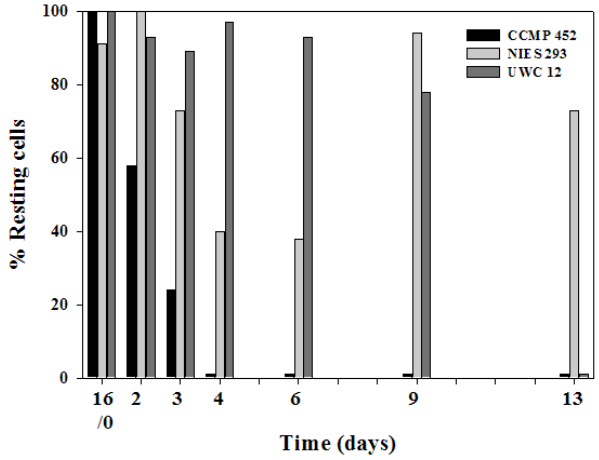

Fig. 2. Percent of resting cells in the cultures of 3 strains of *Heterosigma akashiwo*. Encystment was induced by incubation at 10ºC in darkness for 16 days. Cysts were activated (day-0) by incubation at 20ºC under light (Han *et al*. 2002).

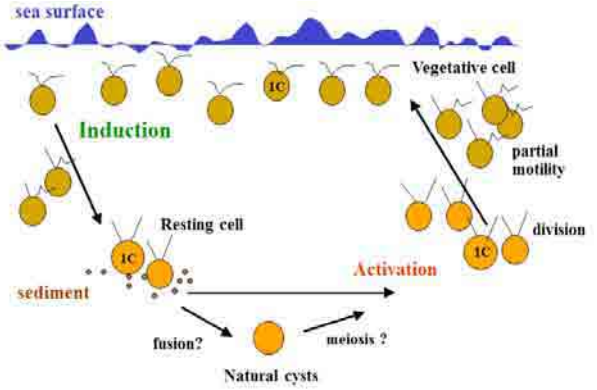

Fig. 3. The hypothetical life history of *Heterosigma akashiwo*. Vegetative cells can be induced by cold temperature and light deprivation to transform into resting cells. Though immobile and unable to divide, the resting cell can survive in stasis for prolonged time periods. Elevation of temperature and restoration of light serve as activation cues that restore division potential and motility. Strains of *Heterosigma* progress though the induction and activation stages with different efficiencies (Han *et al*. 2002).

having low germination rate. As shown in figure 1, the mono-modal blooming species have an incessant cyst- germination strategy. Thus, the cysts of mono-modal blooming species could function as a "seed" rather than a "trigger" for bloom initiation.

Most research of algal cysts has focused on the relationship between germination and algal bloom dynamics. However, high cyst abundance in the sediments is also thought of as a direct trigger for bloom initiation, even if the germination rate is low. Therefore, information on cyst quantification in sediments and studies on the location and size of the "seed bed" are important to obtain a better understanding of the role of cysts in bloom dynamics. However, direct counting methods cannot quantify the exact cyst abundance in the sediments. Recently, a quantitative real-time PCR (qPCR) assay has been widely applied to diverse ecological studies has been regarded as an alternative for the breakthrough of technical bottlenecks. However, the development of a qPCR assay in cyst studies has not been sufficiently proven yet. In the future, a new qPCR assay that provides a more accurate quantification of cysts is needed in order to better understand the role of resting cysts in bloom dynamics.

The impact of endogenous regulation and genetic diversity on cyst germination strategy

As noted in our data, the cysts of some algal species undergo maximum germination under conditions that are unfavorable for vegetative cell growth. We suggested that this germination pattern could be explained by the presence of an endogenous annual clock. The endogenous rhythm should be maintained even as the cell was stored under constant laboratory conditions. For example, Anderson and Keafer (1987) reported on the persistence of the germination rhythm for two years, when algal cysts were held under constant conditions. In our own studies, we found a regular germination rhythm in *A. tamarense*, *S. trochoidea*, and *A. cylindrica* in Korean waters over the course of 2 years (Fig. 1). Tobin and Horner (2011) reported that excystment seemed to be primarily regulated by endogenous factors, and that specific environmental cues, such as temperature, light, oxygen, and cyst resuspension, were secondary factors. Therefore, it was suggested that maximum seasonal germination during the winter season in our study area was probably associated with the endogenous regulation of cyst germination, regardless of the conditions for vegetative growth.

On the other hand, *A. tamarense* blooms were triggered by the germination of hypnozygotes in a Cape Cod salt pond (Anderson and Morel 1979). However, cyst germination may not be the primary trigger for the bloom initiation of this species in Korean coastal waters (Fig. 1-B). Why is the germination pattern different, despite the species, *A. tamarense*, being the same? These results may be explained by genetic differences due to distant

geographical regions. Scholin *et al*. (1994) studied ribosomal RNA sequences and reported that *A. tamarense* isolates from different geographic regions had unique genotypes. Interestingly, Anderson and Keafer (1987) reported that the cyst germination of this species did not have a similar rhythmic pattern in diverse geographic regions. Thus, variability in the endogenous regulation of germination may be due to an underlying genetic variation. If so, this implies that different genetic backgrounds lead to different endogenous clocks and different germination strategies.

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Climate change and eutrophication of coastal waters: Gyeonggi Bay long-term surveys

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Abstract

Coastal eutrophication is controlled by anthropogenic nutrient loading. This research examines the coupling between climate variability and coastal eutrophication, and discusses how eutrophication and climatic stressors are interacting. Using the last five decades (1960-2010) of climatic data and two decades (1992-2010) of nutrient and phytoplankton data in the macrotidal Gyeonggi Bay (>8m), we present evidence that nitrogen concentration and phytoplankton biomass increased two-fold that was significantly linked to increased air temperature (+1.35^{0}C), SST (+1.37^{0}C), and decreased wind velocity (-1.12 ms^{-1}). In GB, climatic and ecological regime shifts, from a cold to warm phase, have been detected in 1990s and 2000s, respectively. GB's cold phase was characterized not by non-eutrophic and non-stratified state, but by low SST, precipitation and high wind state, that favoured bimodal diatom blooms during spring and autumn. Conversely, during GB's warm phase in the 2000's, late winter blooms occurred presumably because winter warming induced higher solar radiation and nutrients, and dominance of pelagic (*Thalassiosira nordeskioeldii*) over tychopelagic phytoplankton (*Paralia sulcata*). Global warming affects precipation patterns that increase nutrient discharge during summer favouring dinoflagellate blooms. Climatic changes and oscillations can, at times, overwhelm nutrient inputs in terms of controlling the onset, duration, and spatial extent of algal blooms.

Keywords: Eutrophication, Climate Change, Cold Phase and Warm Phase

Introduction

Eutrophication of coastal systems has become a significant problem worldwide in response to increased nutrient loading from urban areas and cultivated land areas (Cloern 2001). Nutrient enrichment has stimulated phytoplankton primary production with increasing occurrences of harmful algal blooms (Heisler *et al*. 2008). Climate change likely influences the vulnerability of estuaries to eutrophication in several ways, including changes in mixing characteristics caused by alternations in freshwater runoff, and changes in temperature, sea level, and exchange with the coastal ocean (Najjar *et al*. 2000), although these outcomes may be difficult to detect in the near-term, because they are masked by the inherent 'background' variability of natural systems, and because there are effects resulting from other human induced changes (i.e. eutrophication; Rabalais *et al.* 2009). The objective of this research is to explore the effect of climatic factors and eutrophication on estuarine phytoplankton biomass. We test the hypothesis that both climatic changes and eutrophication can control the onset, duration and spatial extent algal blooms in estuaries.

Materials and Methods

Study Area: Gyeonggi Bay is the largest estuary on the west coast of the Korean peninsula (Fig. 1). It is a broad, shallow system with an average depth of 10 m and maximum 12 m tidal amplitude inducing a strong tidal current (1.2-2.3 and 0.9-1.9 ms^{-1} during spring and neap tides, respectively, (Choi and Shim 1986). The Bay receives 54 million tons of freshwater day^{-1} from the Han River, which passes through the City of Seoul, and 15 million tons day^{-1} from Lake Shihwa, which was artificially constructed in 1994 and which has undergone significant water quality deterioration and biological changes (Choi *et al.* 1997; Park and Park 2000).

Methods: Sea surface temperature data was collected from Korean Oceanography Data Centre at one station (37^{0}28.08''N and 126^{0}35.36''E) during 1960-2010 (with an interruption in 1990-1995). Values for the Pacific Decadal Oscillation were obtained

from an online source (http://jisao.washington.edu/ao). Nutrient data-sets (NO_3-N, PO_4-P) were obtained from an archival data set of the National Fisheries Research and Development Institute. Phytoplankton biomass (Chl *a*) data for 1992-1997 were obtained from environmental research reports released by several institutions (Dong-gu District Incheon Metropolitan City; Incheon Regional Maritime Affairs and Port Office; Sudokwon Landfill Site Management Corporation, Ministry of Construction and Transportation; Ministry of Land Transport and Maritime Affairs, and Korea Airport Corporation). Moreover, the chlorophyll data-set for 2000-2004 and 2006-2007 was collected from reports issued by the Regional Research Centre for Coastal Environmental of Yellow Sea. During 2008-2010, chlorophyll data were obtained from reports by Incheon Meteropolitan City, Incheon Free Economic Zone.

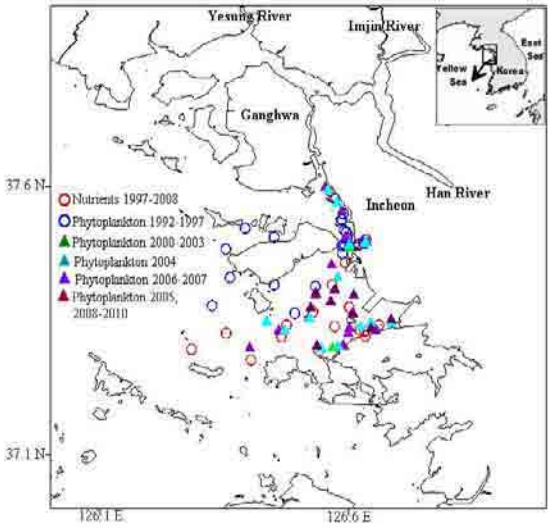

Fig.1: Map of the Gyeonggi Bay showing location of stations for nutrient and phytoplankton biomass

Results

Since 1960, annual mean air temperature in Incheon has exhibited an increasing trend that was most pronounced during the late 1980s (Fig. 2a). Across the Incheon Peninsula, annual mean air temperature had risen 1.35^{0}C during 1960-2010. A SST regime shift had also been detected since 1989, and showed a significant warming trend of 1.37^{0}C since 1964 (Fig. 2c). Gyeonggi Bay became cool during a "warm/positive PDO" with an opposite pattern during a "cool/negative PDO" phase (Fig. 2b) Annual average wind speed over the Incheon region has declined by 23% since the late 1970s (Fig. 2d). An increasing trend of solar radiation and precipitation at Incheon had been detected since 1983 and 1989, respectively (Fig. 2e,f).

Since 2003, GB's nitrate concentration has exhibited a significant increasing trend of about 2-fold (Fig. 3a). In contrast, phosphate concentrations have exhibited an irregular temporal pattern (Fig. 3b). From 1992-1997 to 2000-2010, GB's chlorophyll profiles have exhibited a significant increasing trend about 2-fold (Fig. 3c). Highest chlorophyll levels were recorded in spring, 1998 (7.70 µg l^{-1}) and autumn 1992 (11.40 µg l^{-1}). During the 2000s, different bimodal chlorophyll peaks (winter-summer) occurred. Highest chlorophyll levels were recorded in winter 2001 (20.62 µg l^{-1}), 2005 (16.82 µg l^{-1}), and summer 2005 (12.39 µg l^{-1}). High abundance of nanoplankton chlorophyll was detected during the summer season (Fig. 3d).

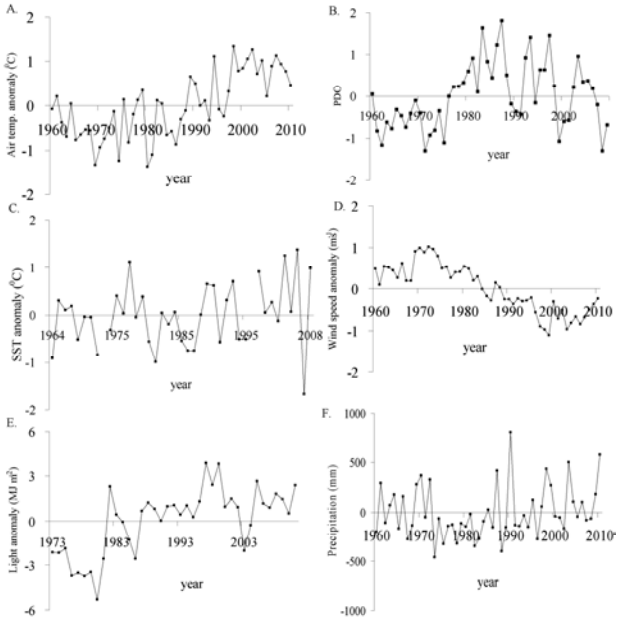

Fig. 2: Annual anomalies of air temperature at Incheon during 1960-2010 (A); Pacific Decadal Oscillation during 1960-210 (B) ; Sea surface temperature in Gyeonggi-Bay during 1964-2009 (C); wind speed at Incheon during 1960-2010 (D); light during 1973-2010 (E); and precipitation at Incheon during 1960-2010(F).

Discussion

SST in GB rose by 1.37^{0}C from 1964-2009. This high rate is consistent with warming in global mean SST (0.67^{0}C) over the last century (Trenberth *et al*.

2007), and in the Yellow Sea (0.67^{0}C) during 1982-2006 and Japan/East Sea (2^{0}C) during 1986-1998 (Belkin 2009). A climate regime shift had been detected since 1989 consistent with North Pacific observtions that a regime shift occurred in 1925, 1947, 1977 and 1989 (King 2005). Winter warming has increased remarkably in GB consistent with warming of the Yellow/East China Sea (Yeh and Kim 2010). GB's warm phases are characterized by decreased wind speed and increased solar radiation, responses consistent with the global trend. Vautard *et al*. (2010) state two probable reasons for decreased wind speed (5-15%) in northern mid-latitudes: (1) changes in mean circulation and/or the decrease of synoptic weather system intensity, (2) changes in near surface wind due to increasing surface roughness (i.e., aerosol). Recently, the global brightening period is reconcilable with changes in cloudiness and atmospheric transmission, and may substantially affect surface climate, the hydrological cycle, glaciers and ecosystems (Wild *et al.* 2005). However, an increasing precipitation trend had been detected since 1989. Nitrogen concentration and phytoplankton biomass increased two-fold during the last decades

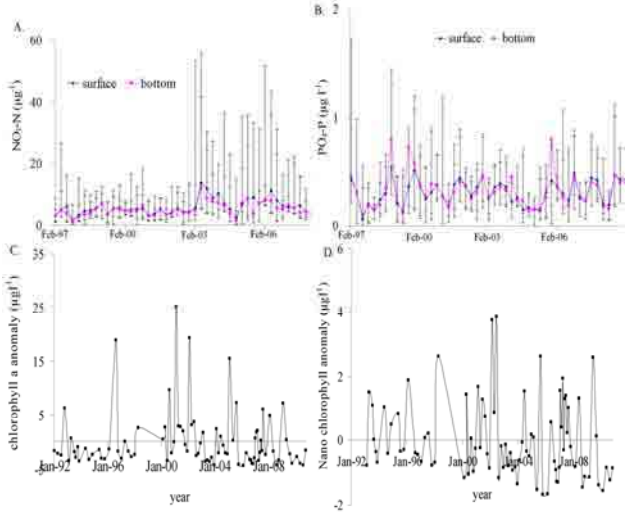

Fig. 3: Long-term variability of nitrate (A); phosphate (1997-2008) (B); chlorophyll a (C); and nanoplankton chlorophyll concentration (1992-2010) (D) in Gyeonggi Bay.

An ecological regime-shift had been detected in the GB since 2000 characterized by bi-modal phytoplankton blooms during winter and summer season. During 1990s' cold phase, the GB's spring bloom was triggered by increasing daily irradiance and atmospheric heat input that stratified the water column after winter mixing and brought nutrient to the surface (Choi and Shim 1986). In contrast, the bloom mechanism in GB during 2000s' winter is a release from strong light limitation by oscillations in other climatic parameters, including increased solar radiation or decreased wind speed, and supports the empirically-based principle that diatoms respond rapidly to episodic high-light, high-nutrient conditions. The dominant species was *Thalassiosira nordenskioeldii* prevailing over the tychopelagic species *Paralia sulcata*. Furthermore, the summer blooms broadly follow a phase I eutrophication model, i.e., nutrient inputs act as a signal and the response to that signal is an increase in phytoplankton biomass (Cloern 2001), with a complex phytoplankton structure dominated by small diatom and non-diatom species such as dinoflagellates, cryptomonads and nanoflagellates. In summer, GB receives huge nutrient loadings through Han River runoff that creates a buoyant surface layer that traps solar radiation, thereby enhancing vertical stability that favours flagellates and single-celled, long-chained diatom (5-50 μm) blooms because they accumulate biomass rapidly following warming and stabilization of a shallow, nutrient-rich surface layer, and tend to aggregate into flocs that sink quickly when nutrients run out (Smetacek 2000). It is therefore concluded that whilst climate is the dominant force affecting annual phytoplankton biomass, eutrophication effects may play an important role in seasonal and community dynamics in Gyeonggi Bay.

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Estimating the effects of ocean acidification-induced behavioral shifts on primary production of *Heterosigma akashiwo*

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Abstract

Primary production rates of the toxic raphidophyte *Heterosigma akashiwo* were estimated when laboratory observations of a pH induced downward shift in vertical swimming behaviors were included. Based on empirical measurements of irradiance, cell distributions and specific primary production rates, total water column production decreased to 1.02×10^{-4} mg C m^{-2} h^{-1} or by 62% due to lower irradiance at depth when pH induced downward swimming was factored into calculations under an ocean acidification scenario. Even inclusion of significantly increased specific primary production rates in a warm and acidified ocean did not negate a decrease in primary production due to downward swimming. If these laboratory predictions apply to the complex conditions in a coastal ocean, the results imply a lower probability of surface slick formation and blooms of *H. akashiwo.*

Keywords: *Heterosigma akashiwo, Ocean acidification, Motility, Population distribution, Primary production*

Introduction

The average pH of the ocean has decreased due to dissolution of excessive atmospheric carbon dioxide (CO_2). The resulting decrease in pH has been termed ocean acidification – a decrease by 0.1 units since the pre-industrial era and a further decrease by a further 0.3 to 0.4 units is predicted by 2100 (Zeebe and Wolf-Gladrow 2001; Caldeira and Wickett 2005; Orr *et al.* 2005). Several studies have investigated the impacts of ocean acidification on various aspects of phytoplankton physiology and ecology, including effects on community composition, calcification rates, photosynthesis, growth rates, and primary production (Kleypas *et al.* 2006; Fu *et al.* 2008; Gao *et al.* 2012).

Changes in the marine carbonate system may also affect harmful algal bloom (HAB) species. For example, Fu *et al.* (2012) predicted an increase in toxicity of some HAB species. Increased growth, carbon fixation and primary production rates have been measured under warm and acidified conditions for the toxic raphidophyte, *Heterosigma akashiwo* (Y. Hada Y. Hada ex Y. Hara et M. Chihara) (Clark and Flynn 2000; Fu *et al.* 2008).

From laboratory experiments, we observed significantly enhanced downward motility in *H. akashiwo* in response to lowered pH (Kim *et al.* 2013). Based on these observations, an advection-diffusion model predicted that at lowered pH the population would undergo a downward shift and fewer cells aggregated at the surface compared to current pH condition (Kim *et al.* 2013). Our study also confirmed prior observation of a pCO_2 fertilization effect on *H. akashiwo* (Fu *et al.* 2008) evident by a 17% increase in growth rates. The effects on primary production rates of a downward shift in the population but higher growth rates in response to lowered pH are unknown.

Here we investigated the relative effects of 1. downward motility, that results in decreased light exposure, and 2. pCO_2 fertilization on total primary production rates of *H. akashiwo*. We found that downward shifts of the population ultimately led to lower estimates of primary production rates for this HAB alga, despite the pCO_2 fertilization effect at lower pH.

Material and Methods

In a prior study, motility parameters including vertical velocity and diffusivity were quantified from 3-

dimensional individual swimming tracks collected in 1L water columns from free swimming *H. akashiwo* cells exposed to pH conditions reflecting pre-industrial (280 ppm), current (380 ppm) and future (750 ppm) atmospheric pCO_2 concentrations (Kim *et al.* 2013). These empirical movement data were used to predict population distributions of *H. akashiwo* as a function of pH treatment using an advection-diffusion model (Kim *et al.* 2013).

Primary production rates were calculated based on two different *H. akashiwo* population distributions: constant cell concentration with depth termed "uniform", and the distribution predicted by our model as a function of pH, labelled according to pCO_2 condition (e.g. "750 ppm"). In addition, we examined the combined effects of higher temperature and 750 ppm termed "greenhouse" on primary production rate estimates.

Calculations used the cellular chl-*a* concentration of *H. akashiwo* (5.23×10^{-6} mg chl-*a* cell^{-1}) from Fredrickson *et al.* (2011) and surface irradiance (E) (µmol photons m^{-2} s^{-1} or µE) of 1250 from measurement in East Sound Washington, USA in July 2007 (Menden-Deuer and Fredrickson 2010). The biomass-normalized primary production rates of *H. akashiwo* was calculated following Jassby & Platt (1976):$P^B(E) = P^B_{max}\tanh(\alpha E/P^B_{max})$, where the photosynthetic performance parameters such as the maximum photosynthetic rate (P^B_{max}) (mgC (mg Chl a)$^{-1}$ h^{-1}) and light utilization coefficient (α) (mgC (mg Chl a)$^{-1}$ h^{-1} (µE)$^{-1}$) of *H. akashiwo* measured by Fu *et al.* (2008) (Table 1).

Table 1. Photosynthetic performance parameters measured at different pH and temperature by Fu *et al.* (2008).

	P^B_{max}	α
380 ppm	1.29	0.018
750 ppm	1.33	0.032
Greenhouse	2.14	0.024

Shading of deeper phytoplankton due to shallower aggregations was not considered. Calculations are detailed in Menden-Deuer (2012). All analyses were performed on MATLAB 7.13. Difference in distributions of primary production rates was examined with the Kolmogorov-Smirnov test (K-S test) and a significance level of 0.05.

Results and Discussion

For uniform with depth *H. akashiwo* distributions and a surface irradiance of 1250 µE, primary production was invariant in most of the water column due to sufficient light availability and slightly higher at higher pCO_2 concentration (Fig. 1). Decay in primary production with depth was only evident at surface irradiance of 150 µE (data not shown). In contrast, when *H. akashiwo* distributions were modelled to be bimodal with a smaller peak at the subsurface and the largest abundance at depth due to pH induced downward swimming, volume specific primary production rates reflected this shift in cell distribution (Fig. 2). Although depth specific primary production rates were significantly different (all $p < 0.001$) between 380 and 750 ppm, the magnitude of the difference was slight (4%).

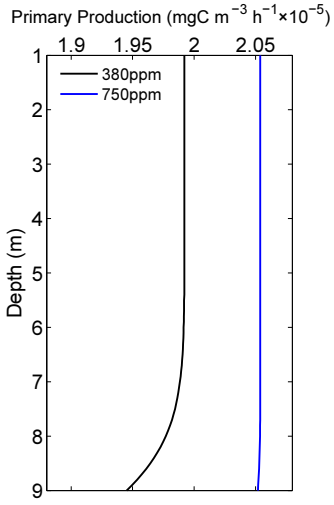

Fig. 1. Vertical profile of primary production (mg C m^{-3} h^{-1}) of *H. akashiwo* in a 9-m water column with surface irradiance of 1250 µE. Predictions were based on a uniform with depth cell concentration.

When estimates were based on downward shifted population distributions, due to pH effects, vertical profiles indicated that primary production was higher at the surface and at 9-m depth compared to uniform distribution estimates because of denser cell concentrations regardless of pH conditions. For instance, in current pH 40.5 and 45.5% of cells were found at the surface and 9-m, respectively. Under acidified condition, this distribution changed to 22.5 and 67.5% at the surface and 9-m, respectively. Although more cells were found in deeper layers, they were exposed to a lower light intensity (~100 µE).

Irrespective of surface irradiance level, the depth-integrated primary production in the water column was highest based on greenhouse condition, whereas

differences between 2 pH conditions were relatively small (Fig. 4). As a function of pH and temperature conditions, primary production was saturated at different light levels. Primary production at greenhouse condition was light saturated at higher surface irradiance (>800 µE), whereas primary production was light saturated at surface irradiance near 500 µE irrespective of pH conditions.

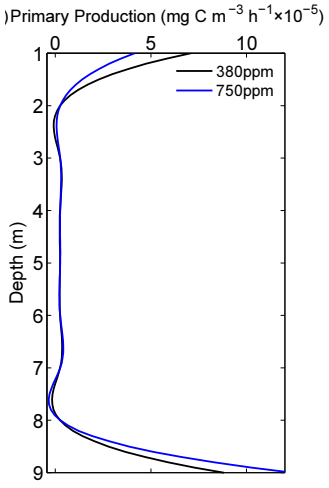

Fig. 2. Vertical profile of primary production (mg C m^{-3} h^{-1}) of *H. akashiwo* in a 9-m water column with surface irradiance of 1250 µE. Surface and depth aggregations resulted from behavioural responses of cells to lower pH.

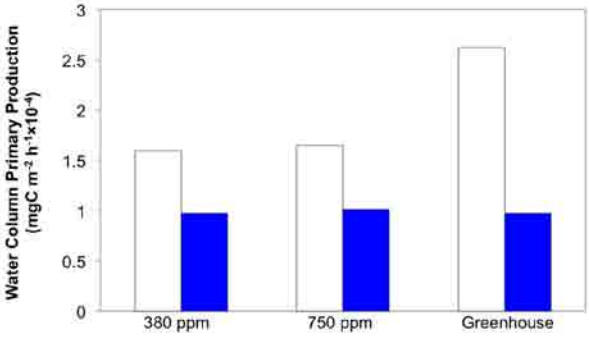

Fig. 3. Integrated water column primary production (mg C m^{-2} h^{-1}) of *H. akashiwo* as a function of pH/ temperature and vertical population distribution, estimated at a surface irradiance of 1250 µE. The rates based on a uniform with depth cell concentrations (white bars) were always higher than the rates based on behavioural responses of cells to lower pH (blue bars).

Thus, downward shifts in population abundance would only reduce primary production rates at surface irradiance of < 500 µE.

Discussion

This analysis showed that behavioural responses to changed pH conditions, leading to a downward shift in population distribution, resulted in a nearly 62% decrease in depth integrated primary production. This decrease is largely due to decreased exposure to light for *H. akashiwo*, which were found deeper in the water column in acidified condition.

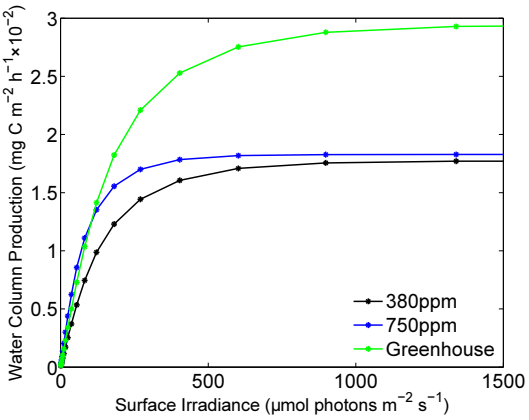

Fig. 4. Relationship of integrated water column primary production estimates (mg C m^{-2} h^{-1}) as a function of surface irradiance levels (µmol photons m^{-2} s^{-1}).

For any of the population distributions, differences in total primary production between current and acidified conditions were not as large as the effects of a shift in population distributions. This may imply that altered behaviour and resulting downward shifted population distributions in decreased pH condition have important ramifications for primary production and bloom formation potential of this HAB species. Primary production via photosynthesis leads to growth and determines growth rates of the bloom-forming alga. A number of studies have reported increased growth rates of *H. akashiwo* under acidified condition (Clark and Flynn 2000; Fu *et al.* 2008; Kim *et al.* 2013), which might be explained based on this alga's absence of a carbon concentrating mechanism (Nimer *et al.* 1997). Fu *et al.* (2008) showed that carbon fixation rates of *H. akashiwo* also increased under acidified condition. This could be interpreted that under future acidified conditions *H. akashiwo* may have a greater growth potential. However, those rates were examined assuming invariant light availability. Given that the population of *H. akashiwo* were shifted towards deeper layers in acidified condition (Kim *et al.* 2013), our finding implies that increased growth rates and primary production

of *H. akashiwo* in decreased pH do not necessarily lead to an increase in primary production. The downward shift resulted in considerable lowering of light exposure, which would be exasperated if we assumed lower surface irradiances. It is noteworthy that in a salinity stratified estuary, cells at depth would also be subject to higher predation pressure (Harvey and Menden-Deuer 2012; Strom *et al.* 2012). Our laboratory analysis of *H. akashiwo* motility was made in the dark over 6 hrs to reduce alterations of ambient pH due to respiration. Thus, we do not know how motility would change in response to prolonged darkness or in alternating light dark regimes. In addition, the assumption used in our study that there is a uniform distribution of *H. akashiwo* in the water column does not take into account natural variability in algal distributions and community structure. Given these limitations, this analysis suggests that anticipated increases in *H. akashiwo* primary production due to increased CO_2 availability are negated by decreases in light availability to cells moving to deeper waters in response to lowered pH.

Application of photosynthesis parameters based on warm and acidified conditions did lead to significant increases in estimated primary production rates. However, a downward shift in population abundance nonetheless lowered estimates by >50%. If applicable to the complex coastal oceans, our results imply a lowered likelihood of the characteristic surface slicks that constitute HAB formation by this raphidophyte alga (Taylor and Haigh 1993). These results make observations of *H. akashiwo* distributions in the coastal ocean and assessment of the relative rates of primary production and grazer induced losses, including in response to manipulated conditions that mimic climate change projections paramount.

Acknowledgements

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Co-occurrence of toxic dinoflagellates *Pyrodinium bahamense* var. *compressum* and *Gymnodinium catenatum* in Acapulco Bay, Mexico

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Abstract

The dinoflagellates *Gymnodinium catenatum* (cosmopolitan) and *Pyrodinium bahamense* var. *compressum* (tropical) are saxitoxin producers. From July 2010 to January 2011 both species co-occurred in Acapulco Bay, located in the southern Mexican Pacific. The density of both species was obtained from bottle samples collected bimonthly (Oct. 2009 to Jan. 2011) and biweekly in July, at different locations and depths in the Bay. Several environmental parameters were evaluated. *G. catenatum* was present all the time with abundances from 20-188.7x10^3cells/L, and low abundances from December to May. At the beginning of the rainy season (July) its abundance increased, declined at the end of the rainy season (Sep.) and increased again in November, with saxitoxin in shellfish reaching 739 µg STXeq./100g. A bloom of *P. bahamense* var. *compressum* was recorded in July 2010, decreasing from August to September, increasing again in November and gradually declining until January 2011. The highest density of *Pyrodinium* (7.73x10^5cells/L) occurred on July 10 at the center of the Bay, with average values of 63.4x10^3cells/L, and saxitoxin values of 104-2092 µg STX eq./100g. Both species showed highest abundances between 3 and 5 m depth. Only *P. bahamense* var. *compressum* showed a positive correlation with phytoplankton biomass (Chl*a*).

Keywords: Toxic dinoflagellates, Mexican Pacific, HAB, *Pyrodinium bahamense* var. *compressum*, *Gymnodinium catenatum.*

Introduction

The toxic dinoflagellate saxitoxin producers: *Gymnodinium catenatum* Graham (cosmopolitan) and *Pyrodinium bahamense* var. *compressum* (Böhm) Steidinger, Tester *et* Taylor (tropical) are common at the Mexican Pacific and producers of HABs (Cortés-Altamirano *et al*. 1995, 2004; Gómez-Aguirre 1998; Gárate-Lizárraga *et al*. 2006; Hernández-Becerril *et al*. 2007; Meave-del Castillo *et al*. 2008; Band-Schmidt *et al*. 2011). In the Americas *Pyrodinium bahamense* var. *compressum* is confined to the south of the Mexican Pacific and Central America (Meave-del Castillo *et al*. 2008).
From July 2010 to January 2011 both species co-occurred in Acapulco Bay, located in the southern Mexican Pacific.

Material and Methods

The study area

Acapulco Bay is located in the tropical Mexican Pacific on the coast of Guerrero state, between 16°35'24" to 17°28'12" LN and 99°25'12" to 110°33' LW.
Samples were collected through the water column with a Van Dorn bottle at 8 locations at different depths: 1, 3, 5, 10 m and bottom, fixed with acetate Lugol. Utermöhl´s chambers were used to count the cells. Sampling was bimonthly from October 2009 to January 2011 and during the HAB of *P. bahamense*, cells were counted in weekly samples (July 10th, 17th, 24th and August 4th).
The sample dates for saxitoxin evaluation in shellfish (*Chama mexicana, Crassotrea gigas* y *Lyropecten nodosus*) were July 7th, 8th, 12th, 19th, 26th and 29th. Toxicity was tested using a mouse bioassay. Temperature, salinity and oxygen were measured with a multiparemetric sensor (YSI-556 MPS); nutrients were analyzed using standard procedures (Solórzano 1969; Murphy and Riley 1962; Strickland and Parsons 1972). Chlorophyll *a* was estimated with a Spectrophotometric method (Parsons *et al*. 1984). From MODIS images of chlorophyll *a* (CONABIO 2011) were analyzed the tendencies of

chlorophyll using biweekly average images.

Results

Abundance. *Gymnodinium catenatum* was present throughout the study period with abundances of 20-188.7x10^3 cells/L, with monthly average values from 128 to 38.3x10^3 cells/L (Fig. 1). During the dry season (December to May) mean abundances were lowest (4x10^3 cells/L). At the beginning of the rainy season (July) the abundance increased to almost 10^4 cells/L, declined at the end of the rainy season (September) and increased again in November (188.7x10^3 cells/L). In January abundance decreased, although in some locations densities were higher than 5x10^3 cells/L.

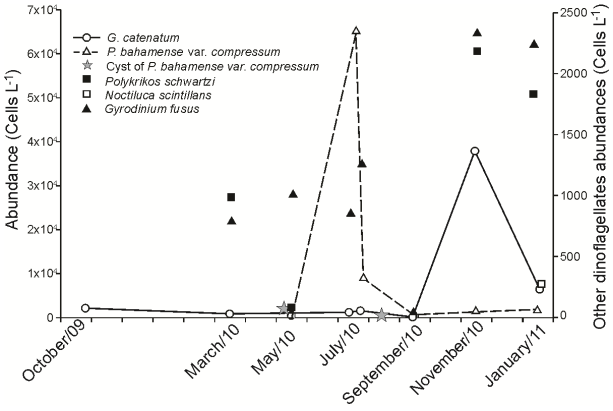


Fig. 1. Average abundance of *G. catenatum*, *P. bahamense* var. *compressum* (vegetative cells and cysts), *Gyrodinium fusus*, *Polykrikos schwartzii* and *Noctiluca scintillans,* in Acapulco Bay from 10/2009 to 01/2011.

Pyrodinium bahamense var. *compressum* was recorded as a bloom in July 2010, decreasing from August to September, increasing again in November and gradually declining until January 2011 (Fig. 1). The event in November could be considered different from the July bloom. The highest density of *Pyrodinium* (7.73x10^3 cells/L) occurred on July 10th, at the center of the Bay, with average values of 63.4x10^3 cells/L.

Both toxic species showed the highest subsurface abundances between 3 and 5 m deep (Fig. 2.)

Saxitoxin.

G. catenatum and *P. bahamense* var. *compressum* are saxitoxin producers. The concentration range of saxitoxin in shellfish *Chama mexicana* was from 104 to 2,092 µg STX eq./100g (Fig. 3). Good agreement between the peaks of saxitoxin and the highest densities of both species was observed, in July with *P. bahamense* var. *compressum* and in November with both species.

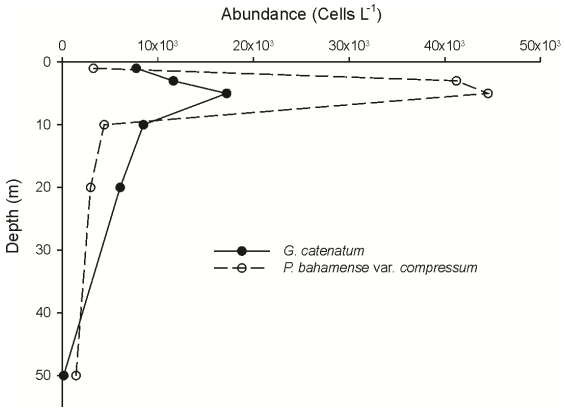


Fig. 2. Average abundance profile of *G. catenatum* y *P. bahamense* var. *compressum* during the study period.

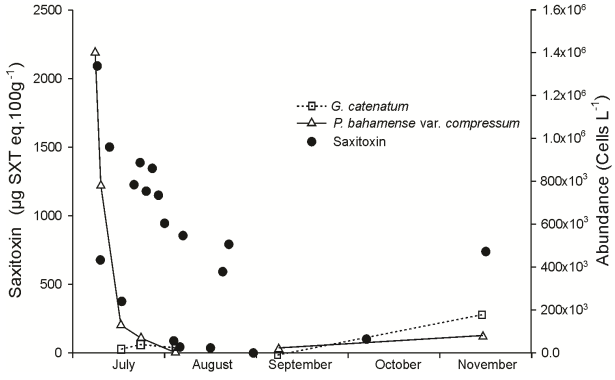


Fig. 3. Maximum values of abundance of *G. catenatum* and *P. bahamense* var. compressum, and saxitoxins concentrations in shellfish.

Morphotypes.

Throughout the study period we found two morphotypes of *P. bahamense* var. *compressum* (one rounded and the other compressed, with a range L/W =0.71-1.16,). The compressed form (L/W<1.0) was always dominant, but when the highest density was recorded, the proportion of rounded cells increased, along with an increase of single cells or short chains (Fig. 4).

Cyst. Both species produce cysts, however in the water column only cysts of *P. bahamense* were found in May, two months before the first vegetative cells, and in August when the bloom declined (Fig. 1).

Predators.

During the bloom of *P. bahamense* and *G. catenatum* the dinoflagellates *Gyrodinium fusus* (Meunier)

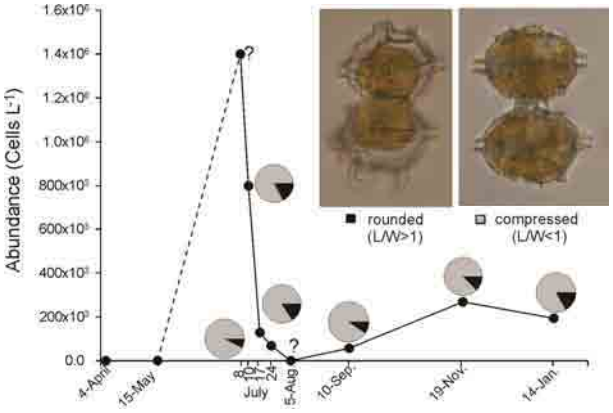
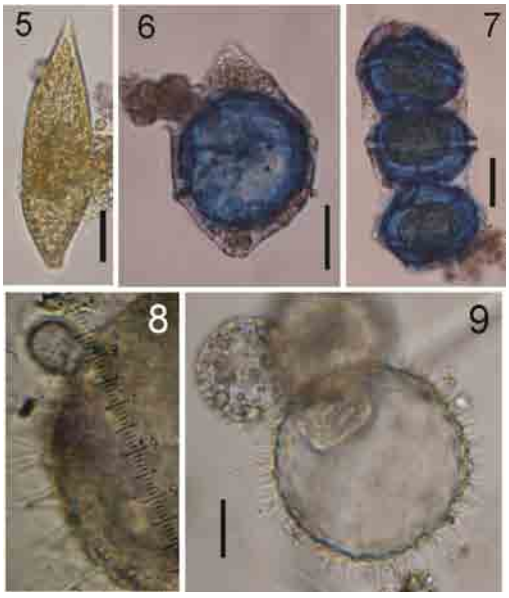


Figure 4. Abundance and proportions of morphotypes of *P. bahamense* var. *compressum*.



Figures 5-9. 5) *Gyrodinium fusus*, 6-7) *G. fusus* (trypan blue stained) with engulf cells of *P. bahamense* var. *compressum*; 8-9) Cyst of *P. bahamense* with trofonts of *Chytriodinium affine*. Scale bars: 5-7, 9 = 20 µm.

Akselman, *Noctiluca scintillans* (Macartney) Kofoid *et* Swezy and *Polykrikos schwartzii* Bütschli increased their abundances (Fig. 1). Since other authors have reported that these dinoflagellates are phagocytes on these toxic species (Holmes *et al.* 1967; Alonso-Rodríguez *et al.* 2005), we can consider that they act as biological controls (Fig. 5-7).

Even cysts of *P. bahamense* were seen with attached trophonts of the dinoflagellate parasite *Chytriodinium affine* (Dogiel) Chatton (Figs. 8-9).

Satellite images.

In February 2010 we observed the presence of an algal bloom in the Gulf of Tehuantepec (Fig. 10a), and during March it moved to the West (Fig. 10b). In May the entire Pacific coast of southern Mexico showed high values of chlorophyll (Fig. 10c). In July the *Pyrodinium bahamense* HAB occurred in Acapulco (Fig. 10d). In October we recorded again an algal bloom in Tehuantepec (Fig. 10e) that moved to the West during November and December (Fig. 10f), producing the second bloom in Acapulco and coastal area of Guerrero and Michoacan States (Gárate-Lizárraga *et al.* 2012).

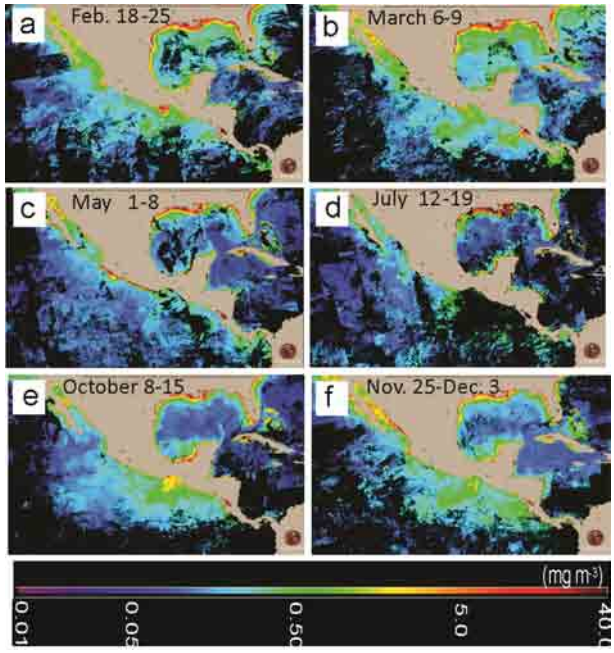


Figure 10. Satellite images of chlorophyll weekly averages of different months of the year 2010.

Relationship of environmental parameters with species abundance.

The results of multivariate analysis including abundances and environmental parameters showed no significant differences. However, the abundance of *P. bahamense* var. *compressum* was correlated with an increase in temperature (Fig. 11) and nutrients in the water column, while for *G. catenatum* the relationship was the opposite. The highest density of both toxic species showed a positive correlation with phytoplankton biomass concentration (Chlorophyll *a*; Fig. 12)

Conclusions

We conclude that *P. bahamense* occurred in Acapulco because cysts were horizontally transported from the southeast region of the Mexican Pacific, where

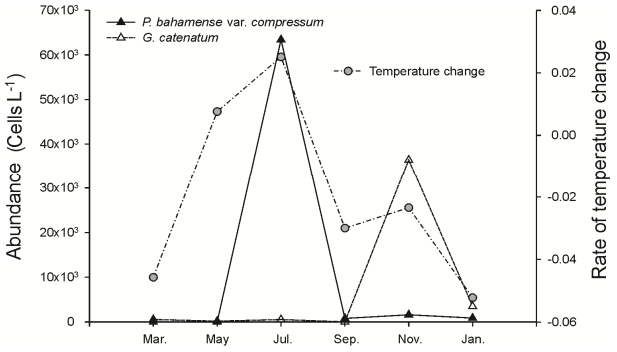


Figure 11. The relationship between the rate of temperature change and maximum abundances of *P. bahamense* var. *compressum* and *G. catenatum.*

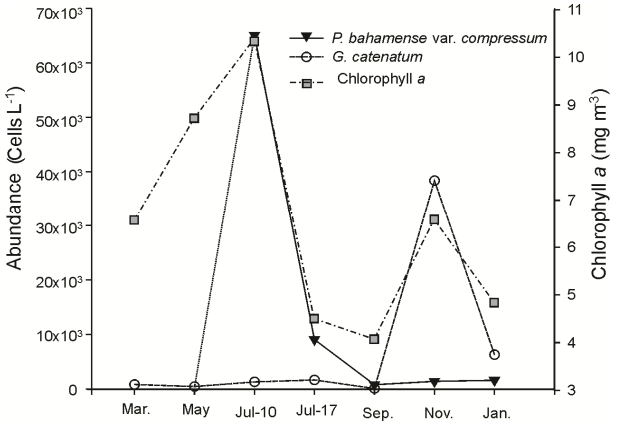


Figure 12. The relationship between chlorophyll *a* and maximum abundances of *P. bahamense* var. *compressum* and *G. catenatum.*

HABs of this species occurred since the begining of the 2010. This assumption is based on the fact that cysts were found in Acapulco in early May and before that time, no vegetative cells were found. In the Pacific Ocean HABs of this species are related with "El Niño" events because these weather conditions cause an increase of water temperature and the occurrence of heavy rains that facilitate the development of *Pyrodinium* possibly in response to increased nutrients and runoff. In contrast, in Mexican Pacific *G. catenatum* is associated with a decrease in temperature and increase of nutrients that occur in Acapulco at the end of the year. In 2010 both species were present in Acapulco because the first part of the year was characterized by the predominance of "El Niño" event and the second part by that of "La Niña".

Acknowledgements

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Feeding behaviour of *Fragilidium* cf. *duplocampanaeforme* and *F. subglobosum* on four *Dinophysis* species

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Abstract

We studied the feeding behaviour of several *Fragilidium* strains of *F. duplocampanaeforme* and *F. subglobosum* from the Atlantic, NW Iberian coast, towards four *Dinophysis* species (*D. acuminata, D. acuta, D. caudata* and *D. tripos*). LC-MS analyses were performed to follow the fate of toxins in the water or their transference from *Dinophysis* cells to *Fragilidium*. Different feeding behaviour and mechanisms were observed among the *Fragilidium* strains: *F. duplocampanaeforme* fed upon *D. acuminata* and *D. caudata* but not on *D. acuta* and weakly on *D. tripos*. *Fragilidium* engulfed these preys directly through the sulcus as previously described. Allelopathic effects toward certain but not all *Dinophysis* species studied were observed. Frequency of gamete-like small forms of *F. duplocampanaeforme* was much higher (>50%) in cultures preying on *Dinophysis* (*D. acuminata* and *D. caudata*) than in those (<20%) where *Dinophysis* cells were not eaten. Our results are discussed in terms of feeding selectivity, grazing rates, life cycle and toxins transfer through the planktonic food web.

Keywords: *Dinophysis*, mixotrophy, *Fragilidium,* okadaic acid, dinophysistoxins

Introduction

Dinoflagellates are marine protists which include photosynthetic and heterotrophic species, but also many mixotrophs (Stoecker 1999). Mixotrophy can be obligatory, like in photosynthetic *Dinophysis* species that "steal" plastids periodically from the ciliate *Mesodinium rubrum* (Park *et al*. 2006). However, in many cases mixotrophic dinoflagellates contain plastids of their own and maximize their growth rates by ingesting other algae (Jakobsen *et al*. 2000; Stoecker 1999; Jeong *et al*. 2005). Mixotrophic dinoflagellates can be found in most taxonomic orders such as Gymnodiniales, Prorocentrales, Dinophysiales, Gonyaulacales, etc (Stoecker 1999).

Fragilidium is a facultative mixotrophic dinoflagellate. The mixotrophic nature of most *Fragilidium* species has been already reported (Park and Kim 2010). According to former studies, *Fragilidium* feeds only on dinoflagellates but distinct selectivity is observed for each species. Thus, *F. subglobosum* feeds only on *Ceratium* spp., *F. mexicanum* on multiple genera (e.g. *Akashiwo, Alexandrium, Ceratium*), and *F. duplocampanaeforme* on *Dinophysis* species (Park and Kim 2010, Hansen *et al*. 2011).

Recently, Park and Kim (2010) described prey specificity and the feeding mechanism in *Fragilidium duplocampanaeforme* isolated in Masan Bay, South Korea. Based on a previous report of *F. duplocampanaeforme* containing *D. acuminata* and *D. caudata* in the French Atlantic coast (Nézan and Chomérat 2009), these authors demonstrated that their isolate fed exclusively on *Dinophysis*. They tested four species: *D. acuminata, D. caudata, D. forti* and *D. infundibulus*. Only *D. forti* was not ingested by *Fragilidium* but allelopathic effects, specifically reduced mobility, were observed in every *Dinophysis* spp.

In the NW Iberian Peninsula, shellfish harvesting closures due to diarrheic poisoning are common in the Galician Rías upwelling coastal system due to the occurrence of *Dinophysis* species, mainly *D. acuminata*, but also *D. acuta* and *D. caudata*. Toxin profiles and cellular content are highly variable between *Dinophysis* species (and between strains of the same species), but all the aforementioned are known to produce okadaates (okadaic acid and its dinophysistoxins, DTX's) and/or pectenotoxins

(PTX's) in some degree (reviewed in Reguera *et al* 2012).
The occurrence of *Fragilidium* spp. is well known in Galician waters, but to our knowledge there are no systematic studies on its abundance, diversity and feeding behaviour on *Dinophysis* spp. or any other dinoflagellates in the area. Amorim *et al*. (2013) described the presence of *F. duplocampanaeforme* and *F. subglobosum* in NW Iberian Peninsula and observed the feeding of both species on *Ceratium horridum*.
In the present study we studied the feeding behaviour of two strains from *Fragilidium duplocampanaeforme* (VGO1120 from NE Atlantic and VGO692 from Mediterranean Sea) and *F. subglobosum* IO97-01 (NE Atlantic), on four *Dinophysis* species isolated in the Galician Rías (*D. acuminata, D. acuta, D. caudata* and *D. tripos*).

Material and Methods

Dinophysis species were isolated in NW Spain (*D. tripos* and *D. acuminata* (VGO1062 and VGO1063, Oct 2009), Station B1, Ría de Vigo; 42º 21,40'N 8º 46,42'W; *D. caudata* (VGO1064, Apr 2010), *D. acuta* (VGO1065, Oct 2010); Station P2, Ría de Pontevedra; 42º 8,22' N, 8º 51,36' W). The ciliate *Mesodinium rubrum* (AND-A0711) fed with the cryptophyte *Teleaulax amphioxeia* (AND-A0710) was added periodically as prey. Cultures of *Fragilidium* cf. *duplocampanaeforme* were isolated during opportunistic samplings at Ría de Vigo, NW Iberian Peninsula (VGO1120; Station B1, July 2009) and Elefsis Bay, Saronikos Gulf (VGO692, July 2003). *Fragilidium subglobosum* (IO97-01) was established by isolation of single cells from plankton-net samples in Portuguese coastal waters. All cultures were grown in diluted (1/20) L1-Si medium at 19ºC, salinity of 32, 12:12 L:D cycle at 150 µmol photons m^2s^{-1} irradiance.
Cultures of *Fragilidium* and *Dinophysis* species were mixed in a 3:1 cell:cell ratio in 24-well microplates (Thermo Scientific, NY, USA).
Instantaneous rates of increase (r; t^{-1}) were calculated using the equation $r = \ln (Nt/N0)/ \Delta t$, and the ingestion rates (I= *Dinophysis* cells eaten *Fragilidium*$^{-1}$ d^{-1}) was calculated following the equations by Frost (1972).
Cultures for toxin analyses were filtered through 1.4 µm Whatman GF/C glass fiber filters. Toxins contained in cells were extracted with MeOH and cell free culture medium was extracted in solid phase (SPE) with Sep-Pak C18 light cartridges (Waters, USA) (Paz *et al*. 2004).
Samples of cells and cell free culture medium were analyzed by LC-MS for lipophilic toxins (YTXs, SPXs, AZAs, OAs, DTX2 and PTX2) at the Analytical Chemistry Department of the University of Vigo following the conditions proposed by Braña-Magdalena *et al*. (2014). These are based on the EC (2011) method validated by the European Union Reference Laboratory on marine biotoxins (EU-RL) under acidic conditions

Results and Discussion

The Atlantic *Fragilidium* (VGO1120) fed selectively and heavily upon *D. acuminata* and *D. caudata* (Fig. 1A, B). Different sizes typically described in *Dinophysis* spp. were distinguished and enumerated in this particular experiment. The aim was to detect if feeding of VGO1120 on *Dinophysis* spp. could discriminate among different cell sizes. But we observed the same trends in their abundance during the experience and only total counts for each *Dinophysis* species are shown in Fig. 1.
Maximum ingestion rates were observed on *D. acuminata* (day 1, I = 2.52 ± 0.48 *Dinophysis Fragilidium*$^{-1}$ d^{-1}) and *D. caudata* (day 3, I = 0.58 ± 0.32). These maximum ingestion rates were similar to those measured in *F. subglobosum* feeding on the dinoflagellate *Ceratium lineatum* (Skovgaard 1996; I=2-4.5).
The Atlantic *Fragilidium* grazed only occasionally on *D. tripos* (I<0.10) and not at all on *D. acuta*. Grazing on *D. tripos* was confirmed by light microscopy after observing the direct engulfment of a few *D. tripos* cells, but never on *D. acuta*. Allelopathic effects (reduced mobility and occasionally cellular death) were observed in all *Dinophysis* species, excepting *D. acuta*.
Higher growth rates and cell densities of the Atlantic *Fragilidium* were measured in *D. acuminata* and *D. caudata* treatments at the end of the exponential phase, reached in <10 days (0.34 d^{-1} in both cases), with lower values if cultured with *D. acuta* and *D. tripos* (0.22 d^{-1} and 0.19 d^{-1}, respectively). The Atlantic *Fragilidium* grew faster when feeding actively on *Dinophysis* during the first week of the experiment relative to the control treatment. After that, cultures reached the stationary phase at day 9. The control treatment of the Atlantic *Fragilidium* reached higher cell densities and the stationary phase at day 13, but corresponding growth rates

were lower (0.17 d^{-1}). At the end of the experiment, dominance of small sized cells in VGO1120 was observed in *D. acuminata* (59.5% ± 3.9) and *D. caudata* (49.8% ± 10.6) treatments, relative to *D. acuta* (19.4% ± 2.7), *D. tripos* (14.9% ± 0.8) and the control treatments (34.1% ± 3.5).

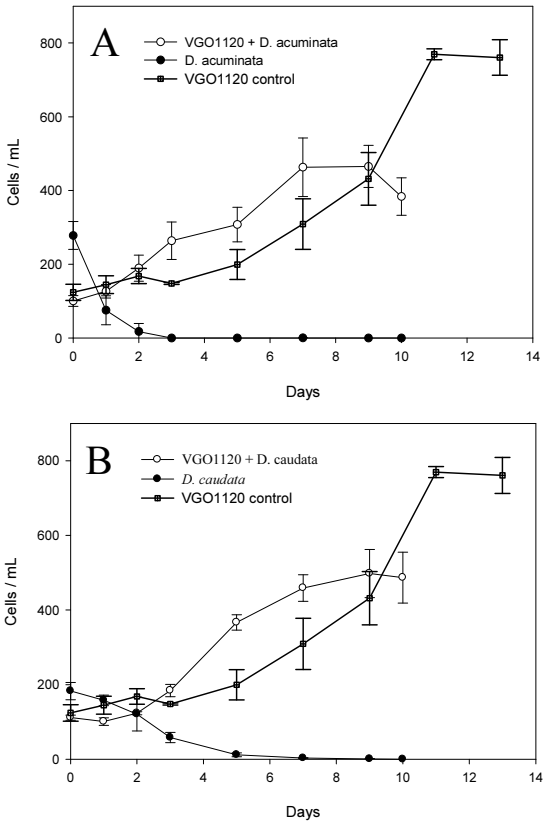

Fig.1. Cell counts of Atlantic *Fragilidium cf. duplocampanaeforme* (VGO1120) cultured with A) *Dinophysis acuminata*, B) *D. caudata*. VGO1120 control treatment and cell counts for each *Dinophysis* species are shown in each plate.

Hansen (2011) recently described *Fragilidium* mixotrophic behaviour as "type-1": "*addition of prey results in large increases in growth rates, at least at low irradiance"*.

In our study a faster initial growth rate was measured in *F. duplocampanaeforme* fed on *Dinophysis*, as reported in *F. subglobosum* fed on *Ceratium lineatum* and *C. tripos* (Skovgaard 1996; Hansen *et al*. 2000). Nonetheless, these growth rates were not markedly high in comparison with control treatments (autotrophic growth). The differences between mixotrophic and autotrophic growth rates in the Atlantic *Fragilidium* could have been larger if higher prey:predator ratios or lower irradiances were assayed.

None of the *D. acuta* cells were ever seen ingested either by the Atlantic isolate VGO1120, or by any of the other two *Fragilidium* strains in this study. Moreover, we could not find any evidence under the light microscope for the feeding of the Mediterranean isolate of *F. duplocampanaeforme* (VGO692) or *F. subglobosum* on any *Dinophysis* species. The only apparent effects were allelopathic, as a higher proportion of *D. acuminata* cells that looked unhealthy and probably dead appeared with VGO692 or IO97-01 relative to the control treatments.

Lipophilic-toxins were also analyzed in the cells and clarified culture medium to follow the fate (i.e. the potential transference and transformation) of these compounds from *Dinophysis* spp. to *Fragilidium* (specifically VGO1120). YTXs, SPXs and AZAs were not detected in *Dinophysis* or in *Fragilidium* species used in this work. Moreover, DSP-toxins (OAs, DTXs and PTXs) were not found in control cultures of *Fragilidium*. *Fragilidium* cells that had been preying on *D. acuminata* and *D. caudata* showed detectable values of okadaic acid (OA) and pectenotoxin-2 (PTX2), in each case (Table 1).

Table 1. DSP toxins in clarified medium and cells of *Fragilidium*. *D. acuta* and *D. tripos* were not cleared out by *Fragilidium* and their cells also contributed to the toxin amount per cell.

	Clarified medium (pg/cell)			Cells (pg/cell)		
	AO (-)	DTX2 (-)	PTX2 (+)	AO (-)	DTX2 (-)	PTX2 (+)
VGO1120 + D. caudata	22	19	297			28
VGO1120 + D. caudata	19	18	304			29
VGO1120 + D. acuminata	66	66		2		
VGO1120 + D. acuminata	67	67		2		
VGO1120 + D. tripos			177			110
VGO1120 + D. tripos			178			116
VGO1120 + D. acuta	78	31	19	8	4	61
VGO1120 + D. acuta	69	32	21	8	5	60

The clarified medium contained higher amounts of these toxins but also different compounds released from the *Dinophysis* cells that could not be detected in *Fragilidium*. In the case of *D. tripos* and *D. acuta* treatments, similar results were obtained both in the clarified medium and in the cells.

Our results suggest that *F. duplocampanaeforme*

fed on toxic *Dinophysis* spp. accumulates DSP-toxins. These toxins seem to be quickly metabolized and the cellular levels are very much reduced in *Fragilidium* a few days after all *Dinophysis* were removed.

We do not know if transformations of DSP-toxins in *Fragilidium* take place, following a similar scheme to that in filter-feeding mollusks; this needs to be addressed in larger scale experiments to detect minor levels of DSP-toxin derivatives. Finally, we suggest that some prey recognition mechanism, rather than toxicity, could explain the selectivity of the Atlantic and Mediterranean *F.* cf. *duplocampanaeforme* isolates toward the *Dinophysis* species tested in this study.

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Differential effects of algal toxins on grazing by the copepod *Acartia tonsa*

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Abstract

Digital holographic cinematography was used to quantify and compare the grazing behavior of free-swimming *Acartia tonsa* exposed to toxic *Karenia brevis* and *Karlodinium veneficum*. Two grazing behaviors were recognized and quantified; both were log normally distributed. "Sampling" behavior was characterized by short duration appendage beatings for less than 100 milliseconds (ms) while "grazing" behavior generated feeding currents and persisted for up to 1,200 ms. In a filtered seawater control, *A. tonsa* only sampled at low frequencies. With the addition of non-toxic food (positive control), sampling increased slightly while grazing increased substantially. On diets of toxic food, sampling increased, but grazing decreased. This decreased grazing activity varied depending on the mode of action of the brevetoxins in *K. brevis* or karlotoxins from *K. veneficum*. These empirical findings were used to inform a stochastic model to evaluate how species-specific effects on grazing behavior influence top-down control of harmful algal blooms.

Keywords: Brevetoxins, karlotoxins, neurotoxins, cytotoxins, top-down control of algal blooms.

Introduction

While there is general consensus on the importance of bottom up (nutrient) effects on harmful algal blooms (Heisler *et al*. 2008), there is a growing body of literature providing species-specific information on the potential for top-down control by metazoan grazers (Waggett *et al*. 2008, 2012; Hong *et al*. 2012). The purpose of this study was to characterize the potential of the ubiquitous, calanoid copepod *Acartia tonsa* to exert top-down control on blooms of the dinoflagellates *Karenia brevis* and *Karlodinium veneficum*. While both of these species are toxic, there are important differences in the toxins they produce.

Karenia brevis contains brevetoxins, cyclic polyether neurotoxins that bind to voltage-gated sodium channels in nerve cells, disrupting normal neurological processes (Nicolaou *et al*. 1998). Karlotoxins, produced by *K. veneficum*, are polyketides with hemolytic, cytotoxic properties acting on cell membranes (Van Wagoner *et al*. 2008; Peng *et al*. 2010). In food removal experiments using *A. tonsa* and *K. brevis*, a food mixture of more than 50% toxic *K. brevis* cells caused grazing to cease (Waggett *et al*. 2012). In contrast, grazing by *A. tonsa* on toxic *K. veneficum* was suppressed, but did not cease. Low level grazing persisted even on 100% toxic *K. veneficum* diets (Waggett *et al*. 2008).

Digital holographic cinematography was used in a follow-up study to compare behavior of free swimming *A. tonsa* fed toxic *K. brevis* and *K. veneficum* versus a non-toxic diet (Hong *et al.* 2012). Observations revealed two behaviors related to grazing. There was a short duration "sampling" behavior lasting <100 milliseconds (ms) that involved little fluid entrainment and a longer duration "grazing" behavior lasting up to 1,200 ms that generated feeding currents

with food particle entrainment (Hong *et al*. 2012). On diets of toxic *K. brevis,* both sampling and grazing diminished rapidly with increasing concentrations of *K. brevis*.

While toxic *K. veneficum* diets reduced both grazing and sampling, grazing behaviors persisted, indicating the intake of cytotoxic karlotoxins did not completely inhibit feeding activity even when the diet consisted of 100% toxic *K. veneficum*.

Using the empirical data from these studies as input parameters, we developed a stochastic model to assess the potential for copepod grazers to affect toxic blooms of these two common bloom forming species. It is important to point out that it was not the intent of this effort to produce a predictive model, i.e., a tool for evaluating management schemes. Rather, the goal was to generate a model to understand the potential algal bloom mechanisms and determine sensitivity of these mechanisms to various abiotic and biotic processes. In a follow up effort, the model presented here will be more fully developed to serve management purposes.

Material and Methods

A stochastic, predator-prey model was developed as a proof of concept that algal toxins alter grazing behavior sufficiently to affect development of harmful algal blooms. Specifically, models of *A. tonsa* fed on mixed diets of non-toxic phytoplankton and toxic *K. brevis* or *K. veneficum* were developed to compare the effects of brevetoxins and karlotoxins on bloom development.

Acartia tonsa feeding and mortality due to the presence of algal toxins were simulated at each time step by the input of randomly selected multiplier factors: the ingestion factor ($Ingestion_{Factor\text{ntp}}$) and the toxin presence factor ($Presence_{Factor\text{tp}}$). The multiplier values for the ingestion factor were 0, 0.5, or 1. The values for the toxin presence factor were 0 or 1.

The model equations follow (see Table 1).

1. $\frac{dZ}{dt} = Growth_z - Mortality_z$ (Z=zooplankton)

2. $Growth_z = \mu_z * Ingestion_{Factor_{ntp}} * Grazing_{ntp} * Z$

3. $Grazing_{ntp} = \frac{NTP}{\kappa_{ntp} + NTP}$

4. $Mortality_z = \left(m_z + Toxin_{presence_{tp}}\right) * Z$

5. $Toxin_{presence_{tp}} = m_{toxin} * \Pr esence_{Factor_{tp}} * \left(\frac{TP}{\kappa_{tp} + TP}\right)$

6. $\frac{dNTP}{dt} = Growth_{ntp} - Mortality_{ntp} - Growth_z$

7. $Growth_{ntp} = \mu_{ntp} * NTP$

8. $Mortality_{ntp} = m_{ntp} * NTP$

9. $\frac{dTP}{dt} = Growth_{tp} - Mortality_{tp} - Ingestion_{tp}$

10. $Growth_{tp} = \mu_{tp} * TP$

11. $Mortality_{tp} = m_{tp} * TP$

The species-specific grazing model para- meters were taken from Waggett *et al.* (2008, 2012) using the ingestion values for 50:50% mixtures of toxic and non-toxic *K. brevis* and 50:50% mixtures of *K. veneficum.* It was necessary to use 50:50% toxic and non-toxic mixtures of *K. brevis* because *A. tonsa* did not graze on mixtures with more than 50% toxic cells. While *A. tonsa* continued to graze on 75:25% toxic to non-toxic and 100% toxic *K. veneficum* diets, albeit at a reduced rate, we decided the comparison of 50:50% mixtures were the most appropriate values for the models.

Results

Model results from the Simecol program in R are in Figs. 1 and 2. *K. veneficum*, the non-toxic species had equivalent growth rates, but were differentially grazed. The biomass of *A. tonsa*, *K. veneficum* and the non-toxic phytoplankton all oscillated in a complex pattern governed by a series of nonlinear feedback interactions (Fig. 1, Sunda *et al*. 2006). In general, as the copepod densities increased, both the non-toxic and toxic food decreased, but often at different rates. Conversely, as the abundance of *K. veneficum* increased, the *A. tonsa* population declined quite substantially. This reduced grazing pressure allowed *K. veneficum* and the non-toxic species to increase, but at different rates. When the entire

model run is considered, the output indicated that *K. veneficum* blooms would be transient and only occasionally reach a biomass equivalent to or exceeding that of the non-toxic phytoplankton (Fig. 1). In contrast, the model run for *K. brevis*, which suppressed grazing to a greater extent than *K. veneficum*, showed less variability. The biomass of the non-toxic phytoplankton community and *K. brevis* were similar for the first half of the run, after which *K. brevis* became progressively more dominant (Fig. 2). This model result predicts that suppressed grazing leads to prolonged *K. brevis* blooms, which are often observed in the field (Table 2). The outcome showed small oscillations in the *A. tonsa* biomass, with the short-term declines being associated with gradual increases in *K. brevis* biomass. The response of the *A. tonsa* population, however, did not follow the expected pattern. Laboratory grazing experiments have shown that as *K. brevis* exceeds 50% of the phytoplankton biomass, grazing ceased. This result would predict a decline in the *A. tonsa* population through time (Waggett *et al.*, 2012). A possible reason the model failed to show a population decline is that it did not account

Table 1. Model parameters

Parameter	Unit	Value (*Kb*,*Kv*)	Source
Zooplankton Growth Rate (μ_z)	day^{-1}	0.2	Roelke 2000
Zooplankton Mortality Rate (m_z)	day^{-1}	0.10	Roelke 2000
Zooplankton Half Saturation coef-ficient: mortality from toxic phyto-plankton (k_{tp})	$\mu m^3 l^{-1}$	610	Roelke 2000
Zooplankton Half Saturation coefficient for non-toxic phyto-plankton (k_{ntp})	$\mu m^3 l^{-1}$	2.11 x 10^9	Roelke 2000
Maximum Ingestion Rate for Toxic Phytoplankton	Ind x day^{-1}	500 x 24hrs, 1,700 x 24hrs	Waggett *et al.* 2012; Waggett *et al.* 2008
Maximum Ingestion Rate for Non-toxic Phytoplankton	Ind x day^{-1}	850 x 24hrs, 2,875 x 24hrs	Waggett *et al.* 2012; Waggett *et al.* 2008
Toxic Phytoplankton Growth Rate (μ_{tp})	day^{-1}	0.25, 0.4	Hardison *et al.* 2012
Toxic Phytoplankton Mortality Rate (m_{tp})	day^{-1}	0.09	Roelke 2000
Non-Toxic Phytoplankton Growth Rate (μ_{ntp})	day^{-1}	0.25	Roelke 2000
Non-Toxic Phytoplankton Mortality Rate (m_{ntp})	day^{-1}	0.09	Roelke 2000
Max Mortality Rate from toxins (m_{toxin})	day^{-1}	1.35 x 10^{-4}, 1.08 x 10^{-4}	Waggett *et al.* 2012; Waggett *et al.* 2008

The feeding behavior simulation were:
$Feeding_{ntp}$ = log normal probability distribution function > 56
$Feeding_{tp}$ = log normal probability distribution function < 61

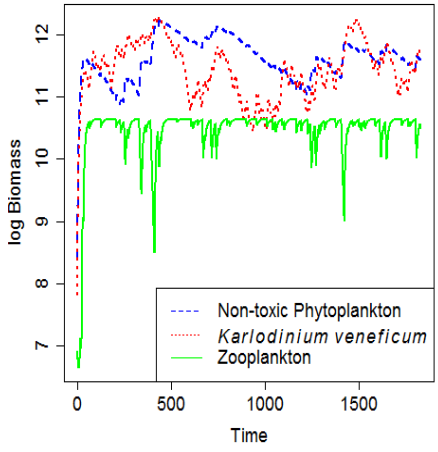

Fig. 1. Model results showing effects of *Acartia tonsa* grazing on toxic *Karlodinium veneficum.*

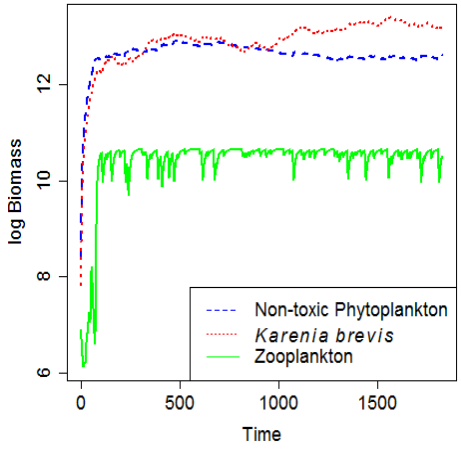

Fig. 2. Model results showing effects *Acartia tonsa* grazing on toxic *Karenia brevis*.

for the effects of reduced grazing on egg production. Tester and Turner (1990) observed *A. tonsa* egg production slows or completely stops within three days of reduced grazing. So, it is likely when reduced egg production is incorporated into the model, a more pronounced decline in *Acartia* abundance will be observed. A more rapid decline in *A. tonsa*, however, would only have accelerated the rate at which *K. brevis* dominated the phytoplankton population. Once the *A. tonsa* population is sufficiently reduced, the length of time *K. brevis* dominates depends on its ability to outcompete co-occurring phytoplankton (Sunda *et al*. 2006).

Discussion

The initial model results indicated that *K. veneficum* blooms would be transient and seldom dominate the phytoplankton community, while *K. brevis* was more likely to form high biomass long-term blooms. These results can, in part, be explained by differences in the modes of action of karlotoxins and brevetoxins. Karlotoxins work by combining with certain membrane sterols, forming pores, and inducing colloid osmolysis to cause cellular breakdown (Place *et al*. 2012). Its primary effect on copepods was to suppress, but not eliminate, grazing. This provided transient relief from top-down control. The modeled effects of altered grazing behavior on the toxic *K. veneficum* biomass were similar to field observations. For example, Hall *et al.* (2008) described a short-lived *K. veneficum* bloom that developed 3-19 October 2006 and collapsed 10-11 days later. Within this short period, *K. veneficum* numbers were as high as 43.5 x 10^6 cells l^{-1}, more than twice the number of the next most abundant phytoplankton species. Hall and co-authors speculated one reason for *K. veneficum*'s dominance was that karlotoxins acted as grazing deterrents and provided a competitive advantage. However, high biomass *K. veneficum* blooms are relatively rare (Place *et al*., 2012) as demonstrated in Fig. 1.

In contrast, *A. tonsa* has no defenses against brevetoxins which directly impair nerve function. Copepod grazing deterrence by these neurotoxins was much greater than for karlotoxins. The initial model results indicated enhanced toxicity would lead to higher biomass blooms of significant duration. These findings were consistent with the observations that *K. brevis* blooms occurred nearly annually in some habitats and can dominate coastal phytoplankton communities for long periods, having escaped top-down control as suggested by the model (Table 2) (Sunda *et al.* 2006).

Table 2. *Karenia brevis* blooms.

Date	Duration (month)	10^6 l^{-1}	Reference
1946	11	56	Steidinger 2009
1998	4	1.12	Vargo *et al.* 2004
1999	6	5.35	Vargo *et al.* 2004
2000	2	2.93	Vargo *et al.* 2004
2001	10	11.23	Vargo *et al.* 2004

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Taxonomy, systematics, and phylogeny

Lectin Binding in Marine Raphidophytes

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Abstract

Lectins are a diverse group of molecules that bind with varying specificity to carbohydrates present on cell surfaces. Since these carbohydrates can play a crucial role in transmitting biochemical signals between and among cells, the carbohydrate composition of cell surfaces is likely to be closely linked to their biology. The marine raphidophytes *Heterosigma akashiwo, Chattonella subsalsa, C. antiqua, Fibrocapsa japonica* and *Haramonas dimorpha* were, along with the haptophyte *Prymnesium parvum* and the dinoflagellate *Gymnodinium instriatum*, preserved in 3 % paraformaldahyde, stained with 13 different fluorescent-labelled lectins and examined under a confocal microscope. *F. japonica* was the only species that tested positive for UEA I and ECL, while only *Chattonella antiqua* tested positive for GSL II. All raphidophytes except *Haramonas dimorpha* tested positive for LCA. We observed substantial fluctuations in binding intensity, even within the same species and a number of clearly distinct binding patterns were evident. We believe the variability in lectin binding patterns reflects the complexity of carbohydrates on the cell surface and may therefore be a valuable tool for species identification.

Keywords: Lectin binding, Raphidophytes, *Heterosigma akashiwo*, harmful algae

Introduction

The Raphidophytes are an important, but not yet well understood, group of unicellular microalgae. As a group they are relatively small, but they are geographically widespread and include both marine and freshwater species. Only the marine genera (*Heterosigma, Chattonella, Fibrocapsa* and *Haramonas*) are included in this study.

It should be noted that *Haramonas'* taxonomic position is uncertain and their definite placement in the Raphidophytes are pending further investigation. Despite low species number they are ecologically important because of their tendency to form dense, harmful blooms many places around the world.

The Raphidophytes are fragile cells which lack hard, protective cell components like theca or frustules, they are further characterized by the presence of multiple chloroplasts located in the periphery of the cell, cell coverings with multiple mucocysts and two heterokont flagella (Hallegraeff & Hara 1995) Hallegraeff & Hara 1995). They also have a glycocalyx, an extracellular coating on the cell surface, consisting of complex carbohydrates and carbohydrate-protein complexes (Yokote et al. 1985) Yokote *et al.* 1985).

Lectins have previously been used to demonstrate the presence of carbohydrates on the surface of protists (Roberts et al. 2006) Roberts *et al.* 2006), microalgae (Costas *et al.* 1993; Rhodes *et al.* 1995; Hori 1996) Hori 1996) and Raphidophytes. (Okamoto et al. 2000) Okamoto *et al.* 2000).

Lectins are a group of biomolecules that traditionally have been isolated from plants but may also include extracts from invertebrate blood. During the last 50 years the number of available lectins has exploded and more than 500 are currently available. They bind to carbohydrate moieties present on cell surfaces with varying specificity. Many lectins are also toxic and may possibly play a dual role in certain organisms; protection from predation and biological recognition molecules (Sharon and Lis 2004) Sharon and Lis 2004).

Due to the large number and varying specificity of the lectins they may represent a new tool for identifying functional groups of microalgae, for example to

Table 1: Lectin overview
Summary of all lectins and species examined. Differentiation has been made between strong (++) and weak (+) positive binding and no binding (-). For the Raphidophytes it is also indicated whether the lectin triggered a response in the cell membrane, resulting in mucus release (M).

	H. aka TX	*H. aka* VI	*F. jap*	*C. ant*	*C. sub*	*H. dim*	*P. par*	*G. ins*
Con A	++ M	++ M	+ M	++ M	++ M	++ M	+	++
WGA	+ M	+ M	+ M	+ M	+ M	+ M	-	+
UEA I	-	-	+	- M	- M	-	-	-
GSL II	-	-	-	+ M	- M	-	-	-
LCA	+	+	+ M	+ M	+ M	-	-	-
DSL	+ M	+ M	+ M	+ M	+ M	+ M	+	+
SNA I	+ M	+ M	+ M	+ M	+ M	+ M	+	+
MAA	+ M	+ M	+ M	+ M	+ M	+ M	-	++
Jacalin	+ M	+ M	+ M	+ M	++ M	+ M	++	+
VVA	-	-	-	-	-	-	-	-
ECL	-	-	+	-	-	-	-	-
DBA	-	-	-	-	-	-	-	-
PNA	+	+	+ M	+ M	+	NA	NA	+

separate toxic from non-toxic species. Differences in the biology of algal cells are likely to be reflected in the carbohydrate composition on the cell surface, which in turn should be detectable by lectins because of their high specificity.

Materials and methods

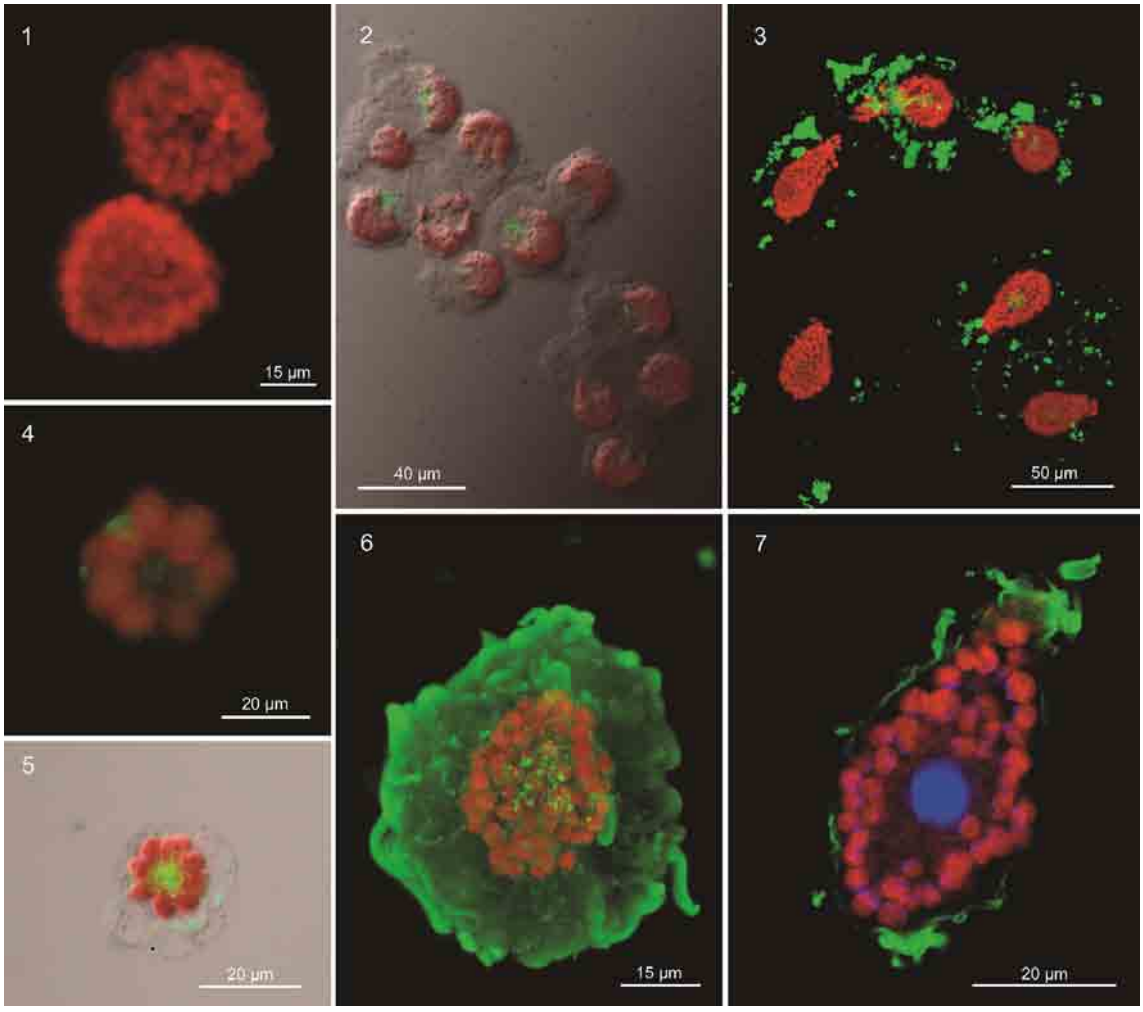
Cultures: All algal strains provided by CMS culture collection, data available upon request.
Fixation and lectin staining: Cultures were grown in dense concentrations and harvested while in exponential growth phase. The algal cells were fixed with 3 % paraformaldahyde in a fixation buffer (480 mM sodium chloride: 14.0256 g/500 ml, 8 mM potassium chloride: 0.29824 g/500 ml, 41.5 mM magnesium sulfate: 5.11425 g/500 ml and 8.25 mM TRIS hydrochloride: 0.6501 g/500 ml in 500 ml dH_2O, pH 8) modified from Yamaguchi *et al* (2010) 2010). Fixative, fixation buffer and algal cells were mixed to 10 ml aliquots and deposited directly on Poly-L-Lysine coated cover slips. Slides were washed 3 x 5 minutes in Sorensens Phosphate Buffer (PBS) pH 7.34, and incubated for one hour, in the dark, with 1% BSA (Sigma-Aldrich) to prevent non-specific binding. Slides were then washed 3 x 10 minutes in PBS and incubated 1 hour in the dark, with the applicable lectin.
All lectins came fluorescent labeled with FITC from the manufacturer, Vector Laboratories (Burlingame, CA, USA) and were used at 10 µg/ml. The exceptions; MAA and SNA-1 were ordered from EY Laboratories (San Mateo, CA, USA) and used at 50 µg/ml. Slides were washed 3 x 10 minutes in PBS. Selected samples were counter stained with DAPI (Invitrogen) to identify DNA. Double stained samples were incubated for 30 minutes in the dark, with DAPI diluted to 300 nM in PBS and washed 2 x 10 minutes with PBS.
Confocal microscopy: Samples were examined with Olympus FV1000 laser scanning confocal microscope, equipped with FluoView software.

Results & Discussion

The amount of fluorescent signal (i.e. amount of lectin binding) was highly variable both between species and between individual cells within a species. Further multiple distinct binding patterns were evident. The relative fluorescent signal strength was estimated and scored as a strong (++) or weak (+) positive signal or no signal (-). Binding of certain lectins to the Raphidophytes also seemed to trigger a response in the cell membrane, resulting in excretion of mucus which formed a characteristic "cloud" around the cell. There was usually strong lectin binding to the excreted mucus which sometimes made it difficult to determine whether there was binding to the surface of the membrane or only to the surrounding mucus. However in some cases mucus was excreted without any evident lectin binding. An overview of all examined species and lectins are presented in Table 1. Positive binding in all species was observed for ConA, DSL, SNA I, PNA and Jacalin. WGA and MAA

tested positive for all species except *Prymnesium parvum.* VVA and DBA were negative for all species; see Figure 1 for example of negative lectin signal. LCA was negative for *P. parvum* and *Gymnodinium instriatum*, but positive for all raphidophytes except *Haramonas dimorpha.* LCA has previously been found positive for *Pfiesteria piscicida* and *Karenia brevis* (Springer 2004), making it an unlikely candidate for raphidophyte detection.

Three lectins showed promise for future rapid detection of two species: UEA I and ECL were negative for all species except *Fibrocapsa japonica* which exhibited low binding in the ventral region of the cell where the mucocysts are concentrated (Figure 2). UEA I has previously screened positive for the toxic dinoflagellat *Alexandrium tamarense* but negative for several other dinoflagellates (Rhodes et al. 1995) Rhodes *et al.* 1995). ECL has also been previously found to be positive for a few toxic dinoflagellates, but nothing morphologically similar to *F. japonica* (Rhodes *et al.* 1995; Springer et al. 2004) Springer *et al.* 2004). No excess mucus formation was visible with either lectin. GSL II was negative for all species except *C. antiqua*, which exhibited bright, patchy binding on the cell surface. Mucus excretion was variable, but patchy bright binding was seen in excreted mucus.



Figs 1-9**:** Examples of Raphidophytes with different binding patterns. 1: *Chattonella subsalsa* stained with VVA (no lectin binding); 2: *Fibrocapsa japonica* stained with ECL; 3: *Chattonella antiqua* stained with MAA; 4: *Heterosigma akashiwo* stained with LCA; 5: *Heterosigma akashiwo* stained with SNA I; 6: *Chattonella subsalsa* stained with Con A; 7: *Chattonella antiqua* stained with WGA and counter stained with DAPI.

As previously mentioned there was surprisingly high variation in binding patterns and intensity. Figure 3 through 7 illustrates examples of binding patterns: Patchy mucus binding involved very strong binding in some areas of the mucus (or on the cell surface) and no binding in others; shown here by MAA binding of *Chattonella antiqua* (Figure 3). The opposite was the homogenous-binding pattern, which exhibited relatively similar binding intensity spread over the whole surface. Figure 4 show low homogenous binding on surface and mucus of *Heterosigma akashiwo* and Figure 6 shows high homogenous binding in mucus surrounding *Chattonella subsalsa*, combined with patchy internal binding. Internal binding was seen in some cases, usually as patchy or centralized in the nucleus area of the cell (Figure 3 and 5). The centralized internal binding was seen with several lectins (WGA, SNA I and MAA) and was possibly enhanced because the cells were fixed before incubation with lectins. A previous study has shown that internal lectin binding enhances in fixed versus live cells (Roberts et al. 2006) Roberts *et al.* 2006). Both lectin-binding and autofluorescence appeared to be transient; when the same specimen was examined at 24 hour intervals, the binding intensity went down with time. Autofluorescence (red signal) faded first, but also lectin binding (green signal) in one week old specimens was notably lower than the first two days after preparation (data not shown), indicating that specimens should be examined within the first two days after preparation. The large variation in binding patterns, intensity and mucus release clearly indicates the complexity of carbohydrates on the cell surface. Differences in cell biology are likely to be reflected in the carbohydrate composition on the cell surface, therefore lectin screening has large potential to separate between functional groups of microalgae like toxic and non-toxic species. This is a technique still in its infancy and much work is still needed before it can be utilized in routine screening.

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Algicidal effects on gene transcriptions of heat shock protein 70 (*HSP70*), *HSP90* and glutathione S-transferase (*GST*) in the dinoflagellate *Prorocentrum minimum*

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Abstract

Algicidal agents are a promising tool to mitigate HABs. These may affect cellular stress gene responses as part of their cell survival strategy. In this study, we investigated the toxic effects of typical algicidal agents, aluminium (Al^{3+}) and the oxidizing biocide chlorine (Cl), on the transcription of stress genes in the dinoflagellate *Prorocentrum minimum*. Test genes included heat shock protein 70 (*HSP70*), *HSP90*, and glutathione S-transferase (*GST*). Both *PmHSP70* and *PmHSP90* were markedly induced by chlorine exposure, but not Al^{3+}. On the contrary, *PmGST* one of the major stress genes was not induced under chlorine exposure, while instead it decreased significantly ($P < 0.01$) under Al^{3+} exposure. These results suggest that both algicides affect *P. minimum* cells in different ways.

Keywords: gene expression, dinoflagellate, algicidal agents, chlorine, Al^{3+}

Introduction

Harmful algal blooms (HABs) are the result of rapid proliferation and high biomass accumulation of toxic or noxious microalgae (Anderson *et al*. 2012). HABs induce mortality in fish, shellfish, and birds, and cause human illness, consequently affecting the aquaculture industry (Harvey and Menden-Deuer 2012). To avoid these impacts, people have tried to control, prevent and mitigate HABs. Algicide treatments are a promising tool for the removal of HABs, however, the mechanism differs with respect to the algicide employed. Some of the algicides, such as copper sulfate ($CuSO_4$) and the oxidizing biocide chlorine (Cl), are very toxic to organisms, and affect cells by oxidative stress (Lopes *et al.* 2001; Russell 2003). Additionally, some algicides, such as Al^{3+}-conjugated compounds, have been used as flocculants to remove HABs (Papazi *et al.* 2009; Vandamme *et al*. 2011).

Dinoflagellates are subjected to various environmental conditions (e.g., thermal shocks, and toxic pollutants), and have evolved elaborate regulatory mechnisms to adapt. Like other organisms, they differ in their gene transcriptions under varying environmental conditions. Even though regulated genes are in low abundance, they can significantly respond under various environmental conditions (Erdner and Anderson 2006; Bayer *et al*. 2012).

Heat shock proteins (HSPs) and other antioxidant compounds can act as protectors, when organisms are exposed to environmental stressors. Among all of the HSPs, the HSP70 and HSP90 family are the most conserved and widely involved in environmental stress regulation of an organism (Sørensen *et al.* 2003). In addition, glutathione S-transferase (GST) is one of the important antioxidants, since they are involved in detoxification and respond differentially to environmental stresses among different prokaryotes and eukaryotes (Blanchette *et al.* 2007; An *et al.* 2012). Among the environmental stressors, algicides cause cellular stress in HABs. However, algicide exposed stress gene responses in HABs have been insufficiently studied.

In the present study, the toxic effects of the common algicides, including the oxidizing biocide chlorine and Al^{3+}, were investigated by analyzing the expression pattern of three stress genes (e.g., *HSP70*, *HSP90*, and *GST*) in the marine dinoflagellate *Prorocentrum minimum*.

Materials and Methods

Cell culture

P. minimum strain (D-127) was obtained from the Korea Marine Microalgae Culture Center (Pukyong National University, Busan, Korea). *P. minimum* cultures were maintained in f/2 medium at 20°C in

a 12:12-h light-dark cycle with a photon flux density of ca. 65 μmol photons/m^2/s.

Stress treatments

P. minimum cultures in the exponential growth phase were treated with algicidal agents. In this study, NaOCl (Cat. No. 425044, Sigma, MO, USA) and $Al_2(SO_4)_3$ (Cat. No. 368458, Sigma, MO, USA) were employed as algicidal agents. To evaluate the effect of the algicidal agents, serial concentrations of each test chemical were added to the cultures, and at predetermined times cell cultures were harvested for cell counts and gene expression analysis.

RNA extraction, cDNA synthesis and gene expression, statistical analysis

Cell cultures were harvested by centrifugation, frozen immediately in liquid nitrogen, and stored at −80°C until RNA extraction. RNA extraction and cDNA synthesis were performed according to Guo and Ki (2012).

Specific primers targeting each *PmHSP70* and *PmHSP90* were designed based on our previous works (Guo and Ki 2012; Guo *et al*. 2012), and used to evaluate the expression of both genes. In addition, partial *PmGST* sequences were retrieved from the *P. minimum* EST database, where the DNA sequences were determined using 454 pyrosequencing (GS-FLX Titanium; 454 Life Sciences, Roche, Branford, CT, USA). The sequence was confirmed by PCR followed by DNA sequencing. We used the determined *GST* sequence to design specific primers: GSTR1- CGA AGG CGG GTC TGC TCA AAT; GSTR2- TTG TGC CGT GCT TGG TCT CAT, which were used for gene expression study. Gene expression and statistical analyses were carried out according to Guo and Ki (2012).

Median effective concentration (EC_{50}) determination

72-h EC_{50} and percentile inhibition were calculated by using cell counts, as recommended in OECD testing guidelines (OECD 2011). The values of 72-h EC_{50} were estimated using a sigmoidal dose-response curve and plotted using Origin version 8.5 (MicroCal Software Inc., Northampton, MA, USA).

Table 1. The effective concentrations (mg/L) after 72-h exposure of algicides to *P. minimum.*

Chemicals	EC_5	EC_{10}	EC_{20}	EC_{50}
NaOCl	0.204	0.437	0.989	1.177
$Al_2(SO_4)_3$	ND	ND	ND	ND

ND, not detectable, due to cell flocculation.

Results and Discussion

Effect of chlorine on the transcription of *PmHSP70*, *PmHSP90* and *PmGST*

In this study, toxic effects of NaOCl to *P. minimum* were assessed. The test species showed a dose dependant decrease in growth rate upon exposure to NaOCl, and its 72-h EC_{50} was calculated as 1.177 mg/L (Table 1). Additionally, EC_5, EC_{10}, and EC_{20} values (Table 1), were calculated to determine the minimum effective concentration required to affect the test species.

For the transcriptional responses of stress genes, the expression levels of *PmHSP70* and *PmHSP90* were induced significantly ($P < 0.05$) by NaOCl treatment (Fig. 1a). The highest relative change of *PmHSP70* was 4.8-fold at 0.1 mg/L of NaOCl compared to the control. At 0.5 mg/L of NaOCl treatment, the increase in expression level of *PmHSP70* occured 6 h after treatment, and gradually increased to 3.9-fold after 24 h compared to the control (Fig. 1c). In the case of *PmHSP90* (Fig. 1a), the highest expression level was observed at 0.2 mg/L of NaOCl treatment, being 4.0-fold compared to control. The increased expression

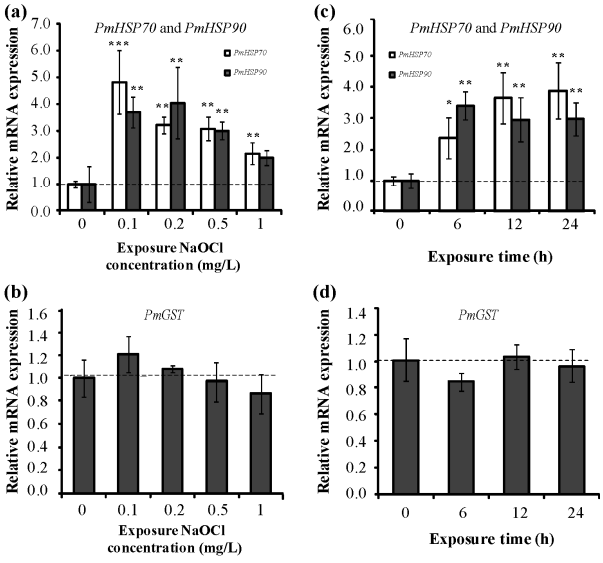

Fig. 1. Relative expression of *PmHSP70, PmHSP90*, and *PmGST* under NaOCl treatments. The gene expression analyses were performed by real-time PCR. (a) and (b): *P. minimum* was treated with different concentrations of NaOCl, and harvested at 24 h; (c) and (d): the cells were treated with 0.5 mg/L NaOCl and harvested at predetermined time. Results are given as the means of triplicates ±SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

level of *PmHSP90* showed similar expression levels after 6 h, 12 h, and 24 h at 0.5 mg/L of NaOCl treatment (Fig. 1c).

As noted previously, the oxidizing biocide chlorine is widely used to control algal cells, and it is also the most effective antimicrobial agent which is widely employed for controlling water quality (Kim *et al.* 2007). Although the mode of action for chlorine is not fully understood, the stress response genes in some targeted organisms can be induced (Bodet *et al.* 2012). For example, a number of stress genes, including stress protein, antioxidant protein, and transcriptional regulator, were significantly induced by chlorine exposure in the bacterium *Legionella pneumophila* (Bodet *et al.* 2012). In *Escherichia coli*, the genes that are involved in the oxidative damage response, cysteine biosynthesis and transport, cold shock protein and outer membrane protein can be up-regulated by chlorine exposure (Wang *et al.* 2009).

Irrespective of varying concentrations of NaOCl and time, *PmGST* was not induced significantly (Fig. 1b).

Addionally, marginal changes in expression of the *PmGST* gene, were observed until 24 h after NaOCl treatment. In the present study, NaOCl was used as the form of oxidizing biocide chlorine. The hypochlorite compounds are considered multitarget reagents that oxidize thiol groups to disulphides, sulphoxides and disulphoxides and have deleterious effects on DNA synthesis resulting from the formation of chlorinated derivatives of nucleotide bases (Russell 2003). As one of the intercellular antioxidants, the glutathione S-transferases (GSTs) play a major role against chemically induced toxicity (Hellou *et al*. 2012). They catalyze the conjugation of glutathione with a wide variety of xenobiotics or reactive oxygen species (ROS) (Prakash *et al*. 2010). However, *PmGST* from the present study was not significantly induced over time with the NaOCl concentration tested.

Effect of $Al_2(SO_4)_3$ on the transcription of *PmHSP70*, *PmHSP90* and *PmGST*

Generally, aluminum (Al^{3+})-conjugated chemicals are used as flocculants to settle down microalgae (e.g., Papazi *et al.* 2009; Vandamme *et al.* 2011). In the present study, we observed that most of the *P. minimum* cells were flocculated with $Al_2(SO_4)_3$ treatment, and thus the effective minimum concentrations for $Al_2(SO_4)_3$ could not be detected. However, according to Yang *et al.* (2013), Al^{3+} is toxic to organisms and produces ROS and reactive nitrogen species. As for the expression of the tested stress genes, *PmHSP70* and *PmHSP90* showed marginal changes, which were similar to the control (Fig. 2a).

In contrast, the *PmGST* expression level was down-regulated (Fig. 2b), and a similar expression level for all Al^{3+} treated samples was observed. It is well known that the GST family members function in chemically induced toxicity defense (Hellou *et al.* 2012), however, different members of GSTs respond differentially to metal stress (Lee *et al.* 2008). The present *PmGST* belongs to microsomal GSTs (unpublished data), which are up-regulated with

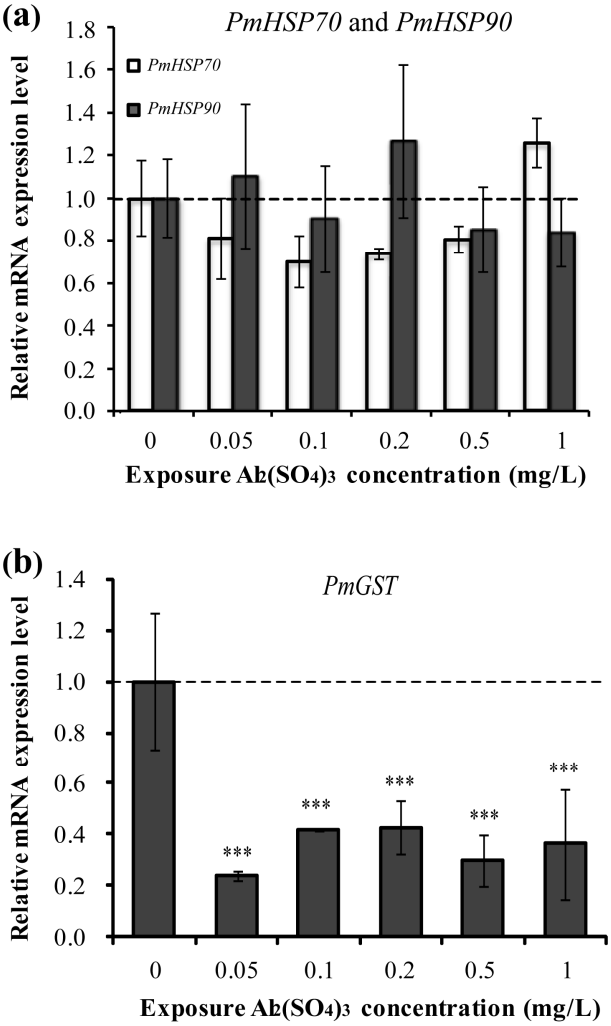

Fig. 2. Relative expression profile of *PmHSP70*, *PmHSP90*, and *PmGST* under $Al_2(SO_4)_3$ exposure. The gene expression analyses were performed by real-time PCR. Results are given as the means of triplicates ±SD. ***$P < 0.001$

metal treatments. For example, down-regulation patterns of microsomal GST were reported for Ag^{+}, As^{+}, Cd^{2+}, and Cu^{2+} treatments in the copepod *Tigriopus japonicus* (Lee *et al.* 2008). These results suggest that Al^{3+} may affect the growth of *P. minimum*, and *PmGST*, but not *PmHSP70* and *PmHSP90*.

In summary, this study provides baseline toxicity data for chlorine and aluminium on *P. minimum*, as well as associated stress gene responses. Furthermore, the tested biocides chlorine and Al^{3+} effectively impacted on growth of the dinoflagellate *P. minimum*, but may differently affect the responses of *PmHSPs* and *PmGST* genes. Further studies are needed to elucidate the differential mechanisms of chorine and Al^{3+} on gene regulation in the dinoflagellate *P. minimum.*

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Growth response of *Pseudo-nitzschia circumpora* (Bacillariophyceae) to different salinities

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Abstract

Pennate diatoms from the genus *Pseudo-nitzschia* Peragallo are known to produce domoic acid and cause Amnesic Shellfish Poisoning (ASP). Although *Pseudo-nitzschia* spp. are commonly found in phytoplankton samples, no ASP has been documented in Malaysia to date. Clonal cultures of *Pseudo-nitzschia* were established and characterized using morphology through electron microscope for ultrastructural analysis. A newly described species, *Pseudo-nitzschia circumpora* was found in four locations in Malaysian waters, indicating the wide distribution of the species. In laboratory studies, *P. circumpora* from Malaysia showed a salinity tolerance from 25-35 psu, with an optimum growth at 30 psu. Further ecophysiological and toxinological studies are needed for a better knowledge of this newly described *Pseudo-nitzschia* species from Malaysia.

Keywords: *Pseudo-nitzschia circumpora*, morphology, physiology

Introduction

Research interests on marine diatom *Pseudo-nitzschia* have risen dramatically after it was confirmed as the causative organism for the first incidents of human intoxication in Prince Edward Island, 1987 (Subba Rao *et al*. 1988). The illness was later known as Amnesic Shellfish Poisoning (ASP) and the species responsible for the event was identified as *P. multiseries* (Bates *et al*. 1989). ASP not only caused poisoning to human beings but also caused death of marine birds and marine mammals in subsequent years (Fritz *et al*. 1992; Scholin *et al*. 2000). Since then, the occurrence of *Pseudo-nitzschia* was well documented worldwide by various research groups (Lelong *et al*. 2012). Studies on the occurrence of *Pseudo-nitzschia* in Malaysia showed a high species diversity with 24 species recorded (Lim *et al*. 2012, 2013; Teng *et al*. 2013). One of these was found to produce high level of DA in cultures (Teng *et al*. 2014). In Malaysia, paralytic shellfish poisoning remained as the biggest concern for the seafood industry and public health due to blooms of the toxic dinoflagellates *Pyrodinium bahamense* (reviewed in Usup *et al*. 2012), *Alexandrium minutum* (Lim *et al*. 2004) and *Alexandrium tamiyavanichii* (Lim *et al*. 2004, 2006, 2007). Since 2009, studies were initiated to document the occurrence, distribution and genetic diversity of *Pseudo-nitzschia* species in order to assess the potential risk of ASP in Malaysian coastal waters. This contribution presents preliminary studies on the ecophysiology of *P. circumpora*.

Materials and Methods

Plankton samples were collected with a 20-µm plankton net. Clonal cultures of *Pseudo-nitzschia* were established using SWII medium (Iwasaki 1961) at 30 psu and maintained under 25ºC, 12:12 light: dark photoperiod with light intensity of approximately 100 µmol photons m^{-2} s^{-1} in a cool-white fluorescence incubator (SHEL LAB, Cornelius, OR, USA). Natural and cultured materials were treated with acid for species identification under transmission electron microscope (TEM). *Pseudo-nitzschia circumpora* was cultured at different salinities ranging from 0-35 psu with sterilized SWII medium; cell densities were enumerated every two days to determine growth rates.

Results and Discussion

In the present study, the stability of morphological

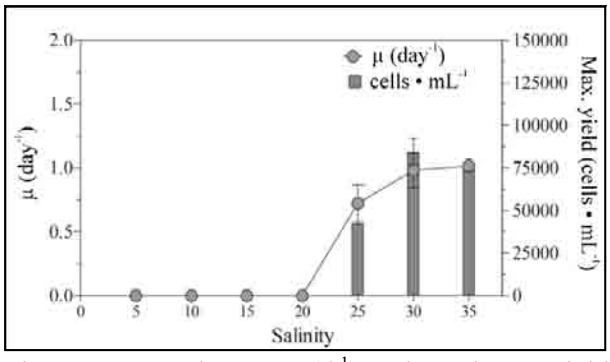

Figure 1. Growth rate, μ (d^{-1}) and maximum yield of *P. circumpora* strain PnSb58 under salinity treatments of 5 - 35 PSU.

characteristics of *P. circumpora* were examined from field samples and resulted with similar morphometric data. Morphometric comparison among the closely related species from the *P. pseudodelicatissima* complex showed that the number of poroids (in 1µm) and of dividing sectors are the most useful and distinctive morphological characteristics to discern *P. circumpora* from the others.

In terms of salinity tolerance, cell divisions were only observed within a salinity range of 25-35 psu (Fig. 1). This explains why *P. circumpora* can only be found in coastal waters of Malaysia but not in more brackish inner waters. No growth was recorded at salinity lower than 20 psu. Cell yield was highest (84,100 cell mL^{-1}) at 30 psu and lowest (<50,000 cells mL^{-1}) at 25 psu. The growth rate (μ) increased with increased salinities from 0.72 d^{-1} at 25 psu to 1.01 d^{-1} at 35 psu (Fig. 1).

The distribution of *P. circumpora* was documented. Only four out of seventeen sampling locations were recorded to have *P. circumpora* and these included: Port Dickson in Negeri Sembilan (Straits of Malacca), Sibu Laut and Bintulu in Sarawak, and Semporna in Sabah.

Future ecophysiology and toxin production studies on this species are essential to enhance our understanding on this *Pseudo-nitzschia* species from Malaysian waters.

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Identification of carbohydrates binding to FITC-conjugated in different species of the genus *Dinophysis*

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Abstract

The identification of *Dinophysis* species is a crucial task in harmful algae monitoring programmes due to the occurrence of several toxin-producing species, agents of diarrheic shellfish poisoning (DSP) events, but with different toxigenic potential. The morphological variability exhibited by some species makes their identification difficult in field samples by conventional light microscopy methods. Molecular analyses of ribosomal genes (18S, 28S rRNA) and their intergenic regions (ITS) have shown limited resolution in this genus due to their low inter-specific variability. The objective of this study was to identify surface carbohydrates, as an alternative to the molecular markers approach, which could be used to identify species of *Dinophysis* from two different localities, Vigo and Huelva, from the NW and SW Atlantic coast of Spain. Our results revealed that *Dinophysis* species used in this study can be identified by their carbohydrate pattern and composition, and that lectins can be used as a complementary tool to molecular studies.

Keywords: *Dinophysis*, FITC-conjugated lectin, carbohydrates

Introduction

Over 100 species of phototrophic and heterotrophic dinoflagellates have been assigned to the genus *Dinophysis* (Gómez 2005). Up to date, about thirteen of these species have been reported to contain potent lipophilic toxins as okadaiates and/or pectenotoxins (Raho *et al*., 2008; Reguera *et al*. 2014). In the Iberian Peninsula, several species of *Dinophysis* are responsible for the highest proportion of shellfish harvesting bans whenever toxins in shellfish exceed regulatory levels established by European directives.

Taxonomic identification of *Dinophysis* spp. is based on morphological features, such as the cell contour and size, and shape of the left sulcal lists (Larsen and Moestrup 1992). But these features cause uncertainty in identification, particularly when two close species of *Dinophysis*, such as the pair *D. acuminata/D. sacculus* co-occur (Zingone *et al*. 1998). Other taxonomic methods, such as molecular analyses based on ribosomal genes and intergenic regions (18S, 28S rRNA and ITS), have shown a limited resolution in this genus due to their low inter-specific variability (Marín *et al*. 2001, Guillou *et al.* 2002).

Lectins are a diverse group of carbohydrate-binding proteins and each of them have their own specificity profile; thereby lectins are suitable for the study of surface-induced events. It is well known that carbohydrate/lectin interactions play an important role in many biological events, and that they can mediate processes such as cell adhesion, cell/cell interactions, glycoprotein turnover, and pathogen recognition leading to innate immune responses.

FITC-conjugated lectin probes have been used to determine the composition of carbohydrates present on the cell surface of many protozoa and dinoflagellates, revealing differences in patterns of lectin binding to the surface (Aguilera and González-Gil 2000). These patterns appear to be species-specific, even when dealing with closely related species (Rhodes *et al*. 1995). Fluorescently tagged lectins were used to discriminate between several harmful dinoflagellates species and can be used as a taxonomic tool to differentiate species (Rhodes *et al*. 1995, Cho *et al*. 1998, 2001).

The objective of this study was to identify surface carbohydrates on different species of the genus

Dinophysis from two different localities in Spain (Vigo and Huelva) as a different approach to methods based on rDNA. The carbohydrate composition of Dinophysis acuminata, D. acuta and D. caudata, was analysed in vegetative cells using ConA, WGA and UEA FITC-labelled lectins, and observed under confocal microscopy.
Our results showed important inter-specific differences in the composition of carbohydrates of the species of *Dinophysis* analysed. In addition, the same species from different localities seemed to have a different lectin-binding pattern.
These results reveal that *Dinophysis* species used in this study can be identified by their carbohydrate pattern and composition, and that lectins could be used as a complementary tool to molecular analyses.

Materials and Methods

Organisms and culture conditions
Cultures of *Dinophysis fortii* were from cells isolated from the coast of Huelva, while those of *D. acuminata* were from Ria de Vigo. Cultures of *D. acuta* and *D. caudata* were from strains isolated from the two localities. The ciliate *Mesodinium rubrum* (AND-A0711) fed the cryptophyte *Teleaulax amphioxeia* Hill (AND-A0710) was added periodically to all *Dinophysis* cultures as prey. Ciliate and cryptophyte strains were isolated in 2007 within the Andalusian Monitoring Programme (Huelva, SW Spain).
The organisms were grown in L1 medium (Guillard and Hargraves, 1993), under 100 µmol m^2 s^{-1} (photosynthetically active radiation), with a 12:12 h dark: light cycle at 20 ºC.
Lectin binding and DNA staining
Three FITC-lectins were used in this study: wheat germ agglutinin (WGA), concanavalin A (ConA) and lectin UEA (Table 1). Lectins were prepared from stocks of 1mg/ml in deionized water. For labelling tests with FITC-conjugated lectins, cells were collected by centrifugation at 3200 g for 5 min, resuspended in 100 µl of sterile artificial seawater with or without lectin (200 µg ml^{-1}) and incubated for 1h in the dark at room temperature. Then, cells were washed for 5 min with sterile artificial sea water and fixed with formaldehyde (4%) for 1h at 4 °C. Thecae were stained with calcofluor (100 µg ml^{-1}) and DNA with 1.5 mM propidium iodide. Preparations were mounted on slides with Prolong Gold (Invitrogen). Slides were observed by confocal laser microscopy (CLSM, Confocal Laser Scanning Microscopy) at 63X, using a Leica TCS SP5 spectral confocal microscope. To test carbohydrate specificity, microalgae were incubated with selected competitive carbohydrates (α-man 100mM, α-L-fucosa 250 mM, β-glcNAc 500 mM, α-galNAc 300 mM). Following incubation (30 min at 21°C), microalgae were washed three times with sterile artificial sea water, labeled with FITC-conjugated lectins as described above, and analyzed by CLSM.

Table 1. Carbohydrate specificity of the lectins.

Lectin	*Specificity*
Canavalia ensiformis (ConA)	α - D-methyl-mannose
Ulex europaeus (UEA)	α-L-fucose
Triticum vulgaris (WGA)	N-acetyl-β-D-glucosamine

Results

Lectin labelling of dinoflagellate cells
First, the affinity and specificity of the FITC-conjugated lectins was tested by incubating *D. acuminata* cells with the lectins and their specific carbohydrates (Table 1). Like the untreated cells, these cells showed no fluorescence (data not shown). To evaluate the involvement of carbohydrates on the microalgae cell surface, cells of *Dinophysis* species were treated with either seawater or a commercially available FITC-lectin. The binding and label position of the lectin on the surface were used as an initial determining factor. FITC-Con A lectin bound to all *Dinophysis* species cells tested (Fig. 1) revealing the presence of α-D-methyl-mannose and α-D-methyl glucose. The strains from Huelva showed a strong, uniform binding, whereas the fluorescence was weaker in those from Vigo and didn´t appear distributed around the entire cell, showing a more discrete binding in the epitheca in *D. acuta* and *D. caudata*. The FITC-UEA binding was stronger in the strains from Vigo, especially in *D. acuminata* and *D. caudata*, indicating the presence of L- fucose, while cells from Huelva exhibited no binding at all, with the exception of *D. caudata*, that showed a very dim fluorescence. All cells treated with FITC-WGA showed fluorescence, revealing the presence of N-acetyl-β-D-glucosamine, although it was stronger in the strains from Vigo those from Huelva. Bbinding in *D. acuminata* and *D. caudata* from Vigo was

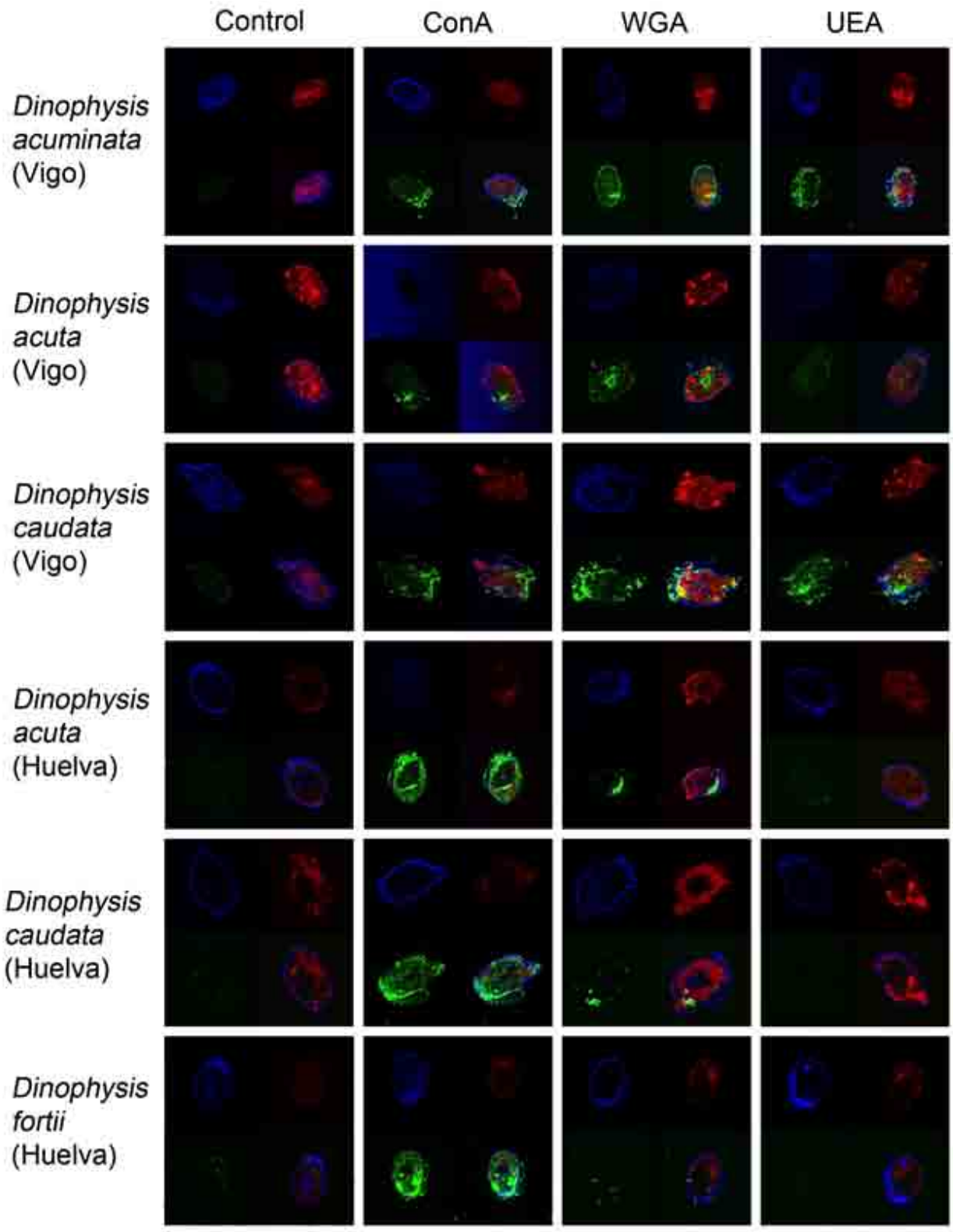

Fig.1 CLMS micrographs of vegetative cells of different *Dinophysis* species from Galicia and Huelva stained with FITC-conjugated lectins (green fluorescence). The thecae was stained with calcofluor (blue fluorescence) while dinoflagellates DNA was stained with propidium iodide (red fluorescence).

around the entire cell, while in *D. acuta* from Vigo and in all specimens from Huelva, the binding showed a more discrete pattern.

Discussion

Dinophysis HABs cause substantial economic losses due to long shellfish harvesting bans. Identification and quantification of this species is needed in HAB monitoring programs. Efforts have focused on the development of techniques for quick and easy identification of *Dinophysis* species to complement that based on morphology.

Lectins are differentiated by their specific carbohydrate affinity at the cell surface and have been used as probes for the identification of morphologically similar species (Costas and Rodas 1994; Rhodes *et al.* 1995; Cho *et al.* 1998, 2001), indicating that fluorescence-tagged lectin probes are potential tools for identification of different species in HAB monitoring programs. Our preliminary results suggest that the combinations of fluorescence-tagged lectins and their different binding activity can be used to differentiate not only different species of *Dinophysis*, but also strains of the same species from different localities. Nevertheless, the use of lectins may present a series of inconvenient. Binding patterns may differ due to genetic variability resulting from geographical isolation and environmental factors. Costas *et al*. (1993) suggested that although asexual cell division had no influence on the lectins binding activity, gametes might do. Aguilera and González-Gil (2000) reported that the cell surface composition may change during the cell cycle in *Alexandrium minutum*, *Gymnodinium catenatum*, *Prorocentrum micans* and *Gyrodinium impudicum* Given that *Dinophysis* spp. has a polymorphic cell cycle, it is important to address if gamete cells have a different lectin-binding patterns when compared to vegetative cells. Further experimental work is required to investigate the relationship, if any, between those factors and the lectin binding patterns in *Dinophysis* species. This is the first report describing qualitatively the cell wall composition of different *Dinophysis* species.

Table 2. Differential lectins labelling in the microorganisms used in this work

Organism	Lectin		
	ConA	UEA	WGA
Dinophysis acuminata (Vigo)	++	++	++
Dinophysis acuta (Vigo)	+/-	+	++
Dinophysis caudata (Vigo)	+	++	++
Dinophysis acuta (Huelva)	++	-	+
Dinophysis caudata (Huelva)	++	+/-	+/-
Dinophysis fortii (Huelva)	++	-	+/-

Acknowledgements

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Does the pinnatoxin-producing dinoflagellate, *Vulcanodinium rugosum*, comprise a species complex?

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Abstract

Pinnatoxins are members of the toxic cyclic imine group and were first characterized over two decades ago. However, the dinoflagellate producer of pinnatoxins was not known until 2011 when cultured from sediment samples in New Zealand. The causative species, *Vulcanodinium rugosum* Nézan & Chomérat, was described in 2011 from a bloom sample from a Mediterranean lagoon. *V. rugosum* has now been recorded around the world including Australia, New Zealand, Japan, China, Hawaii and Europe. *V. rugosum* strains of various origins produce different pinnatoxins and also differ from each other in partial large subunit rDNA and internal transcribed spacer regions suggesting the existence of a species complex. This study sought to determine the status of this species by combining DNA sequence data, morphological characteristics, mating experiments and toxin profiles from strains originating from five geographical locations.

Keywords: *Vulcanodinium*, dinoflagellate, molecular genetic taxonomy, morphology, life-history.

Introduction

Vulcanodinium rugosum Nézan & Chomérat is a peridinoid dinoflagellate recently described from field samples from a Mediterranean lagoon in France (Nézan and Chomérat 2011). Morphologically similar cells were also germinated from division "cysts" isolated from sediments collected in northern New Zealand and South Australia (Rhodes *et al*. 2010, 2011a). From cultures it was determined that this species produces analogues of pinnatoxins (cyclic imines) which were first characterized from shellfish nearly two decades previously (Rhodes *et al.* 2011b).

Since its description and isolation *V. rugosum* has been found worldwide including Japan (Smith *et al.* 2011), China (Zeng *et al.* 2012), and Hawaii (Rhodes *et al.* 2011b). Analogues of pinnatoxin have also been detected in shellfish and water samples in many locations including Scandinavia (Rundberget *et al.* 2011), eastern Canada (McCarron *et al.* 2012), and the Cook Islands (L. Rhodes unpub. data). Thus, *V. rugosum* potentially occurs in most temperate, sub-tropical and tropical habitats worldwide.

V. rugosum has typical peridinoid motile cell morphology. Unfortunately, this cell type is difficult to obtain both from cultures and most field samples except under certain conditions. This has made it difficult to define the morphological characteristics of this species. The motile cells transform readily into the dominant non-motile life-cycle stage – dark brown, spherical, unornamented, non-calcareous division cells (30–35 μm diameter) (Rhodes *et al.* 2011a, 2011b; Zeng *et al*. 2012). These cells divide and form clumps of cells that can produce large amounts of mucilage. Motile cells are then released from these non-motile cells (Rhodes *et al.* 2011a) and tend to be released en masse (L. Rhodes, pers. obs.) under as yet to be determined conditions, although it appears related to the end of a dark period. The sexual reproduction of this species is unknown but it is possible that sexual reproduction does occur to form true resting cysts.

There exists variation in the type and amount of toxins produced amongst the strains. Japanese and French strains both produce pinnatoxin G only (Nézan and Chomérat 2011; Smith *et al.* 2011). New Zealand strains isolated at present do not produce pinnatoxins G, only E and F (Rhodes *et al.* 2010). The Australian strains produce G, E and F, although this varies amongst isolates (Rhodes *et*

al. 2011a). A recent isolate from China produced a new compound that is currently being characterized (Zeng *et al.* 2012).
Due to the differences between the geographic strains we sought to determine if these isolates belong to the same species or are part of a species complex.

Methods

Clonal cultures from New Zealand (CAWD163, 166–8, 170–72, 178), South Australia (CAWD180–3), Japan (CAWD188 and 190), Hawaii (CAWD194) and China (CAWD198) are maintained in the Cawthron Institute Culture Collection of Micro-algae. Light microscopy (LM) and Scanning Electron Microscopy (SEM) analyses for all strains were carried out as described previously by Rhodes *et al.* (2010).
To determine if the strains are genetically compatible and also to establish evidence of a sexual life-stage in this species, dual strains, in all combinations, were grown in the wells of 24-well tissue culture plates (Becton Dickinson). All combinations were grown in GP, modified GP 50%, L1, F2, and K media with and without P and/or N. Different temperature regimes (20°C and 25°C) were also tested. All cultures were observed regularly for >25 weeks.
DNA sequencing was carried out as described by Smith *et al.* (2011) using the primers D1R-F (Scholin *et al*. 1994) and D3B-R (Nunn *et al*. 1996; large subunit ribosomal (LSU) gene); ITS1 (modified from Gottschling *et al.* 2005) and ITS4 (White *et al.* 1990; internal transcribed spacer (ITS) region); 1F and 1528R (Tillmann *et al.* 2009; small subunit ribosomal (SSU) gene); DINOCOX1F and DINOCOX1R (Stern *et al*. 2010; cytochrome *c* oxidase I (COI) gene); and Dinocob4F and Dinocob3R (Lin *et al*. 2009; cytochrome *b* (cob)). Phylogenetic analyses and divergence estimates (uncorrected pairwise distances) were carried out according to Smith *et al*. (2011). Available DNA sequences from GenBank for *V. rugosum* (accession numbers: HQ622102 and HQ622103) were also included in the analyses.

Results and Discussion

All strains of *V. rugosum* examined in this study were morphologically indiscriminable and as described previously in Nézan and Chomérat (2011), Rhodes *et al*. (2011b), Smith *et al*. (2011) and Zeng *et al.* (2012). Mating trial experiments provided no evidence of gametes or sexual resting cyst life-stages.
DNA sequences from mitochondrial (COI, cob) and nuclear (LSU, SSU, ITS) genes were obtained from strains from all geographic locations. Mitochondrial DNA sequences for both genes were identical for all strains. However, all phylogenetic analyses for nuclear DNA sequences showed three clearly defined clades (data for LSU analysis shown only; Fig. 1.). Sequences from New Zealand, South Australia, and the South China Sea all grouped within a well-supported clade (100% posterior probability). The sequences from Hawaiian and Japanese strains formed a moderately supported (54.7% posterior probability) sister clade and a third clade was comprised of the single sequence from the Mediterranean type strain. These clades were also supported by phylogenetic analyses of the nuclear SSU and ITS sequences (data not shown).
Divergence estimates for nuclear genes were calculated using pairwise distances. *P* values for ITS sequence divergence are shown in Table 1. The ITS region has been proposed as a candidate gene for DNA barcoding studies of dinoflagellates (Litaker *et al.* 2007). It has been recommended that sequences from the ITS region varying by $p>0.04$ be used to identify species (Litaker *et al*. 2007).
Values higher than 0.04 would allow the identification of most species, including potentially cryptic species. For the strains examined in this study, within clade variation at the ITS region was very low ($p=0.01$; Table 1.) while variation between the clades was very high ($p=0.09$ to $p=0.13$). These values are well above the suggested threshold for species differentiation of $p>0.04$.
The extremely large variety of forms and evolutionary diversity of dinoflagellates often make species classification very difficult. Additionally, many

Table 1. Divergence estimates as calculated by pairwise distances (*p* values) for the ITS region from all geographic strains of *V. rugosum*. Abbreviations: NZ/SA, New Zealand/South Australia; SC, South China Sea; JA, Japan; HA, Hawaii; EU, Europe.

	NZ/SA	SC	JA	HA	EU
NZ/SA	-				
SC	0.01	-			
JA	0.12	0.13	-		
HA	0.12	0.13	0.01	-	
EU	0.09	0.11	0.11	0.11	-

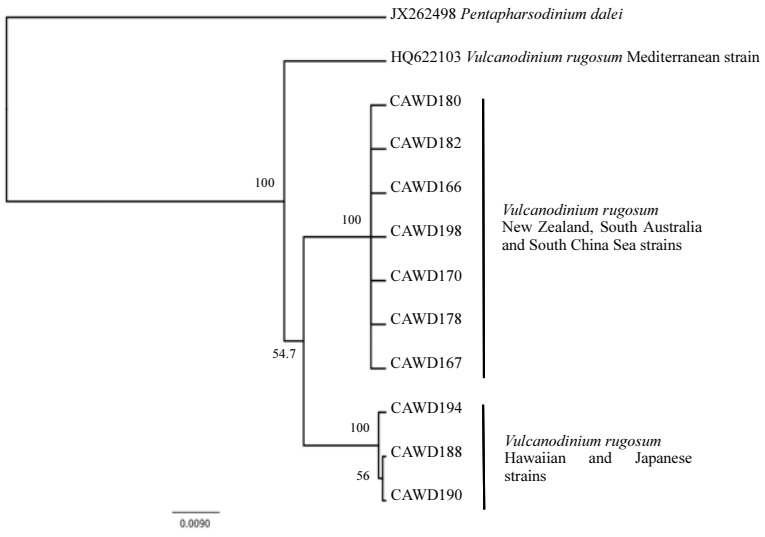

Figure 1. Bayesian phylogenetic analysis of large subunit ribosomal DNA sequences obtained from *Vulcanodinium rugosum* strains maintained at the Cawthron Institute and from GenBank. Numbers on nodes denote posterior probability values. *Pentapharsodinium dalei* was used as an outgroup.

groups of unicellular dinoflagellates are lacking in defining characteristics and this has resulted in considerable taxonomic ambiguity (e.g. Zinssmeister *et al.* 2011). In recent years studies have tried to determine the ideal gene for standard DNA-based identification (or DNA barcoding) system for dinoflagellates (e.g. Litaker *et al.* 2007; Stern *et al.* 2010, 2012). It was determined in several studies that the ITS region can be a suitable marker for defining dinoflagellate species (e.g. Litaker *et al.* 2007; Stern *et al.* 2012). However, it is advised that additional markers are used to avoid inflated diversity due to pseudogenes and paralogues, and to ensure accurate identification (Stern *et al.* 2012). Previous work has also shown that mitochondrial genes are useful DNA barcoding genes for dinoflagellates (Lin *et al.* 2009; Stern *et al.* 2010) with the exception of some groups. In this study both the COI and cob genes showed no diversity within and between the geographic strains sequenced. Clade structure does not seem to be related to geographic location. For example, the strain isolated from South China Sea belongs to the same clade as strains from New Zealand and South Australia, while the Japanese strains form a clade with the Hawaiian isolate. Potentially this distribution could reflect introductions via ballast water. *V. rugosum* has been identified in samples collected from ships' ballast tanks (Garrett *et al.* 2011) and the dominant non-motile cell would increase the likelihood of this species surviving transportation. Morphology-based taxonomy has proved very difficult for many groups of dinoflagellates. On occasion species descriptions have only occurred after molecular identification, e.g. *Gambierdiscus* spp. (Litaker *et al.* 2009). In this study we found three distinct genetic clades within morphologically indiscriminable strains of *V. rugosum.* One clade contains strains from New Zealand, South Australia and South China and one clade contains strains from Japan and Hawaii. The European sequence is currently the only available strain from the original type species and comprises a third clade. Isolates from additional locations will be sought, and morphological characters and life-history traits will continue to be investigated to confirm the genetic groupings.

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Pseudo-nitzschia (Bacillariophyceae) in Malaysia: a record of taxa from field investigations

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Abstract

Different diatoms species of the genus *Pseudo-nitzschia* have been associated with Amnesic Shellfish Poisoning (ASP). In recent years, domoid acid contamination in seafood has been increasingly reported in Southeast Asian countries. To assess the potential risk of ASP and determining species diversity of toxic or potentially toxic *Pseudo-nitzschia* in Malaysia, plankton samples were collected from 17 locations throughout the coasts of Malaysia using a 20 µm-mesh plankton net. Samples were subjected to acid wash treatment prior to observation under transmission electron microscope (TEM). Identification of *Pseudo-nitzschia* species was based on the frustules morphology and morphometric measurements. In total, 22 species were identified, including 14 new records in Malaysia. Occurrence of nine known toxic species which were previously associated with ASP events worldwide are documented. This study reports for the first time a high species richness of *Pseudo-nizschia* along the coastal waters of Malaysia.

Keywords: *Pseudo-nitzschia*, Malaysia, Malacca Straits, South China Sea

Introduction

Amnesic shellfish Poisoning caused by shellfish contamination with a neurotoxin, domoic acid (DA), was first reported at Prince Edward Island, Canada in 1987; the causative organism for the event was identified later as *Pseudo-nitzschia multiseries* (Bates *et al*. 1989). Research on *Pseudo-nitzschia* have gained wide attention after this incidence and numerous studies have been conducted worldwide. Thus far, 37 species of *Pseudo-nitzschia* has been documented (Lelong *et al*. 2012, Lundholm *et al*. 2012), with a few species known to be cosmopolitan (Hasle 2002).

Contamination of domoic acid in shellfish mollusks in Southeast Asia has been confirmed in at least two countries from the region: Vietnam (Dao *et al.* 2009) and Philippines (Takata *et al*. 2009). However, the status of ASP or domoic acid levels in shellfish from neighboring countries remained unclear. *Pseudo-nitzschia* species are common in the phytoplankton assemblages in Malaysian waters. Five *Pseudo-nitzschia* species have been recorded from northeastern Borneo, i.e. *P. brasiliana*, *P. calliantha*, *P. delicatissima*, *P. micropora*, and *P. multistriata* (Skov pers. comm.; Larsen and Nguyen 2004) but there is a lack of supporting information. Recently, the presence of these species, with new records of *P. cuspidata*, and *P. dolorosa* and *P. pungens* (Lim *et al*. 2012) was confirmed. A new morphotype was described as *P. circumpora* based on morphological and molecular evidence (Lim et al. 2012) Lim *et al.* 2012). However, these studies were limited to few locations in Borneo. Hence, a study on *Pseudo-nitzschia* distribution was initiated in 2009 to document the presence of species of this genus along the Malacca Straits and South China Sea. This study was also aimed to assessing the potential risk of ASP in Malaysia, in particular in areas with extensive shellfish mariculture.

Material and Methods

Plankton samplings were conducted at 17 locations along the coastal waters in Malaysia particularly the areas with shellfish farming industry (Figure 1). Samples were collected using 20 µm-mesh plankton net and preserved with acidic Lugol's solution. Plankton samples were rinsed with distilled water before acid washed to remove organic material. Cleaned plankton samples were mounted on the Formvar-coated copper grid, air-dried, and examined

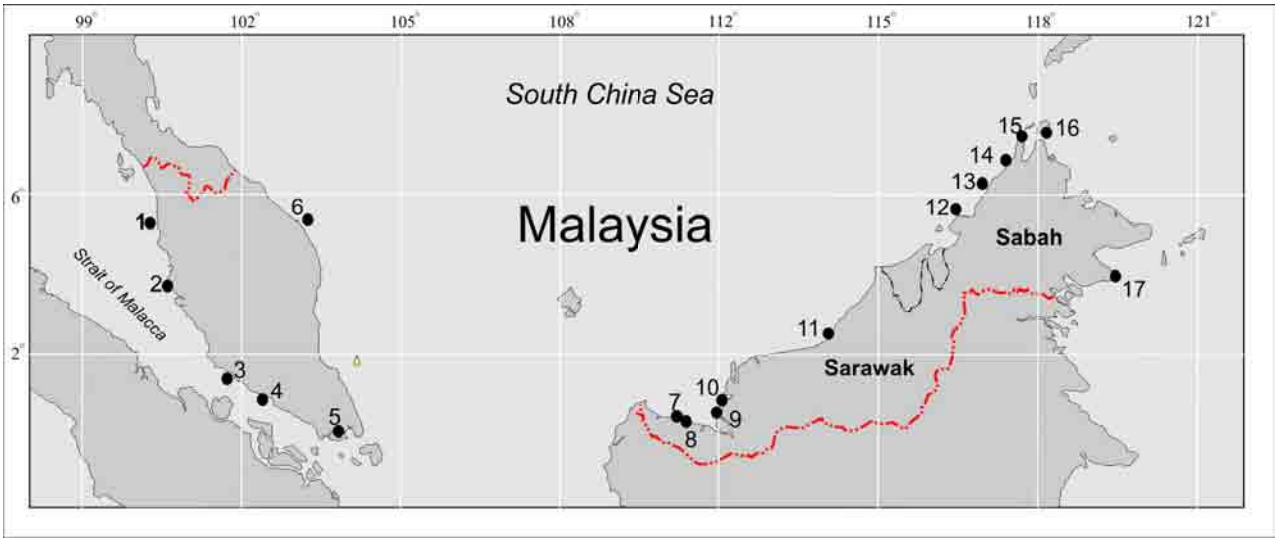

Fig. 1: Sampling locations along the Malaysian coast.

under a JEOL JEM1230 transmission electron microscope (Tokyo, Japan).

Results and Discussion

In this study, a total of twenty two species of *Pseudo-nitzschia* were documented from samples collected in locations shown in Table 1. Generally, at least two potentially toxic *Pseudo-nitzschia* species were observed at each station. Locations such as Port Dickson (Station 3), Bintulu (Station 11), Pulau Banggi (Station 16) and Semporna (Station 17) were suggested as potential ASP hotspots, with at least five known toxic species found in the samples (Table 1). Bintulu recorded the highest diversity of *Pseudo-nitzschia*, with nine known to be toxic, and seven non-toxic *Pseudo-nitzschia* species; it was followed by Port Dickson, with 13 species, nine of which are known to be toxic (Table 1). Five and six potentially toxic species were recorded at Pulau Banggi and Semporna respectively (Table 1).

Table 1: *Pseudo-nitzschia* species found at each samplig location.

Sta.		Species	Total # species	Potential toxic species	Potential ASP risk
1	Queen Bay, Penang	b,r	2	2	Low
2	Teluk Batik	a,b,n,r,t	5	2	Low
3	Portdickson	a,b,d,e,h,i,j,k,n,o,p,r,t	13	5	High
4	Muar	b,r,	2	2	Low
5	Johore	a, b, c, g, r	6	3	Low
6	Kuala Terengganu	b, r	2	2	Low
7	Santubong	b, r	3	3	Low
8	Semariang	b, r	2	2	Low
9	Kabong	b, r	2	2	Low
10	Gerigat	b, r, t	3	2	Low
11	Bintulu	a, b,c,d,e,f,g,h,j,k,l,m,n,,p,r,t,u	16	9	High
12	Kuala Penyu	b,i,l,r	4	2	Low
13	Kota Belud	b,f,q,r	4	4	Low
14	Kota Kinabalu	b,g,h,r	4	3	Low
15	Kudat	b,c,r	3	3	Low
16	Pulau Banggi	b,c,d,e,g,h,i,k,n,r,s,t	11	6	Medium
17	Semporna	b,d,e,g,h,i,k,r,u	9	5	Medium

a. *P. americana;* b. *P. brasiliana c. P. caciantha* d. *P. calliantha.* e. *P. circumpora* f. *P. cuspidata* g. *P. decipiens* h. *P. delicatissima /arenysensis* i. *P. dolorosa* j. *P. hasleana* k. *P. inflatula* l. *P. linea* m. *P. lineola* n. *P. mannii* o. *P. micropora.* p. *P. multistriata* q. *P. pseudodelicatissima.* r. *P. pungens.* s. *P. sinica.* t. *P. subfraudulenta.* u. *P. turgidula.*

Thirteen species from the *delicatissima*-group belonging to three main complexes, *viz. pseudodelicatissima*, *delicatissima* and *americana* complex, were documented in this study. Eight species from the *pseudodelicatissima* complex were observed including three species, i.e. *P. caciantha*, *P. calliantha*, and *P. pseudodelicatissima* (Fig. 2), known to produce domoic acid or associated with ASP events elsewhere (Álvarez *et al.* 2009, Moschandreou et al. 2010) Moschandreou *et al.* 2010). Four species from the *delicatissima* complex (Fig. 2), with only *P. delicatissima* reported as a toxic species (Larsen and Nguyen 2004), were documented. Three species from the *americana* complex (Fig. 2), of which only *P. brasiliana* is reported as toxic (Sahraoui et al. 2011) Sahraoui *et al*. 2011), were also described.

Only three species from the s*eriata*-group, i.e., *P. pungens*, *P. turgidula* and *P. subfraudulenta,* were documented (Fig. 2). Among these, *P. pungens* and *P. turgidula* are known to be toxic (Rhodes et al. 1996) Rhodes *et al*. 1996). Interestingly, species such as *P. turgidula*, *P. lineola*, and *P. decipiens,* previously reported from cold climate regions (Almandoz *et al*. 2008, Marchetti *et al*. 2008, Lundholm *et al*. 2012) were observed in this study (Fig. 2). Further studies are needed to clarify the origin of these species.

Conclusions

Our results showed high diversity of *Pseudo-nitzschia* species in Malaysian coastal waters, with 14 new records in the country. Coexistance of the known toxic and non-toxic species was found in most of the sampling site. At least two toxic or potentially toxic species was documented at each station. Five locations with high numbers of toxic

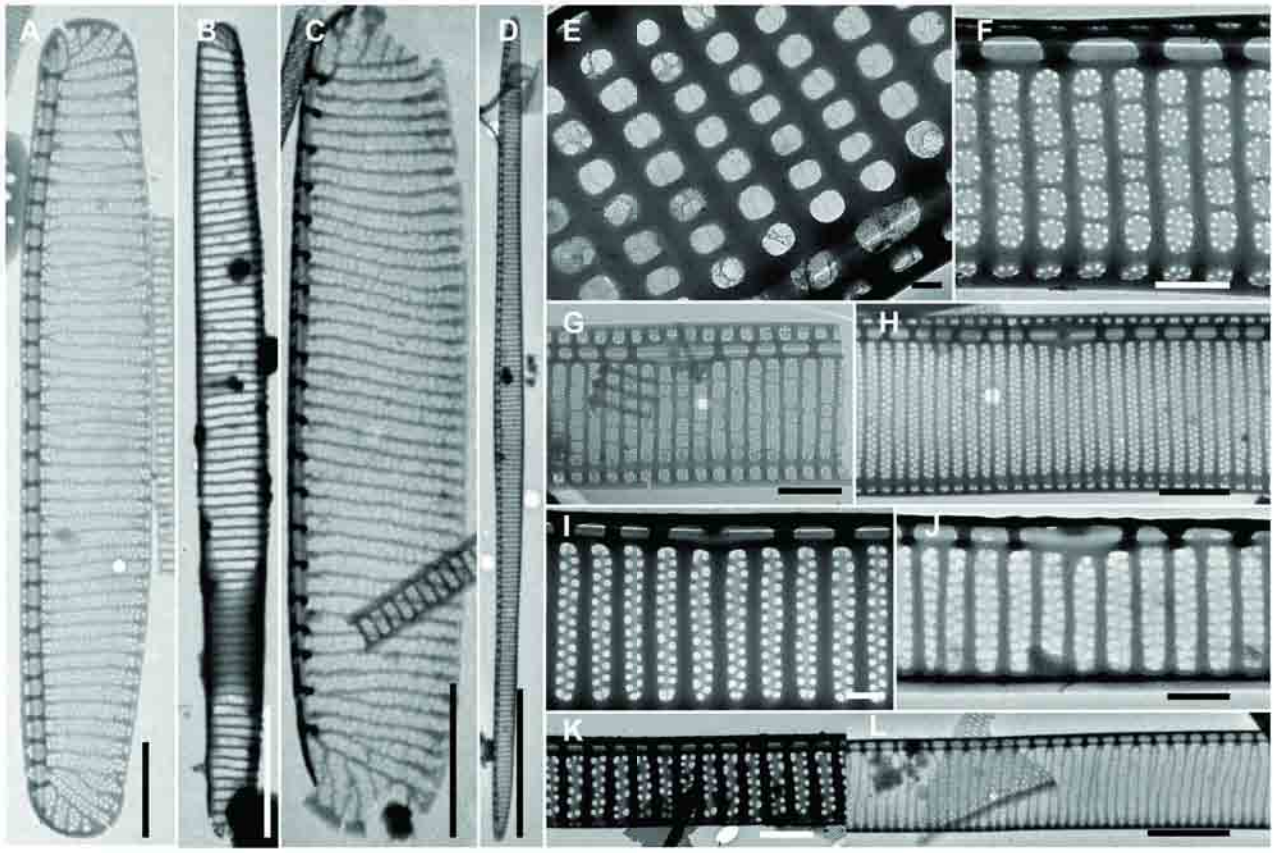

Fig. 2: TEM micrographs of selected *Pseudo-nitzschia* spp. found in this study. (A) *P. amaricana*, linear to lanceolate valve, (B) *P. brasiliana,* lanceolate valve, (C) *P. linea*, linear valve, (D) *P. inflatula*, inflated at tip of the valve, (E) *P. pseudodelicatissima,* two hymen sector, (F) *P. caliantha*, 6-8 sector with central sector, (G) *P. circumpora*, >7 sector in one hymen poroid, (H) *P. subfraudulenta*, 2 rows with dividing sector poroid perstriae, (I) *P. turgidula*, detail of two row poroids, (J) *P. delicatissima*, detail of two row poroid, (K) *P. pungens*, detail of two row poroid, (L) *P. multistriata*, 2-3 row of poroids per striae.

species are potential hot spot area for ASP outbreak and required. Toxin analysis on culture and nature samples is required to study the toxicity of the species in Malaysia. Development of molecular probe for rapid detection of the toxic species is undergoing.

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The dinophycean genus *Azadinium* and related species − morphological and molecular characterization, biogeography, and toxins

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Abstract

Azaspiracids (AZAs) are the most recently discovered group of lipophilic marine biotoxins of microalgal origin. It took about twelve years from the first human poisoning event until a culprit for AZA production was unambiguously identified and described as a novel species, *Azadinium spinosum*, within a newly created genus. Since then, knowledge on the genus has increased considerably, and an update on the current circumscription of the genus is presented here including various aspects of morphology, phylogeny, biogeography, and toxin production. There are currently five described species: *A. spinosum*, *A. obesum*, *A. poporum*, *A. caudatum*, and *A. polongum*. As indicated by molecular sequence variation detected in field samples, there are probably more species to recognize. Moreover, *Amphidoma languida* has been described recently, and this species is the closest relative of *Azadinium* based on both molecular and morphological data. *Amphidoma* and *Azadinium* are now grouped in the family Amphidomataceae, which forms an independent lineage among other monophyletic major groups of dinophytes. Initially, azaspiracids have been detected in *A. spinosum* only, but AZA production within the Amphidomataceae appears complex and diverse: A new type of azaspiracid, with a number of structural variants, has been detected in *A. poporum* and *Amphidoma languida*, and AZA-2 has now been detected in Chinese strains of *A. poporum*.

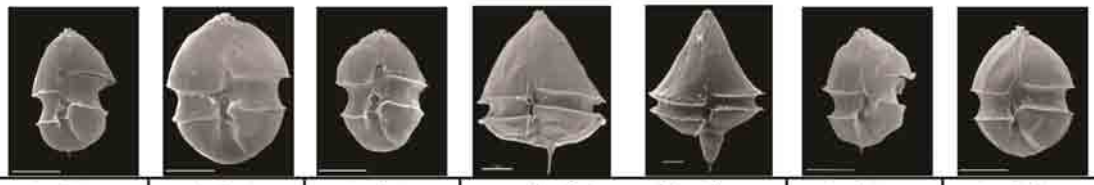
Keywords: Azadinium, azaspiracids, Amphidomataceae, shellfish toxin

Introduction

Among the known marine toxins responsible for shellfish contamination, azaspiracids (AZAs) are the most recently discovered group of lipophilic compounds of microalgal origin. Compared to the knowledge on toxin structure, detection methods, and toxicology, convincing clarification of the aetiology of azaspiracid-poisoning was seriously lacking for quite a long time. AZA toxins are known for their seasonal and episodic accumulation in suspension-feeding shellfish (Salas *et al.* 2011) − a situation similar with several other marine biotoxins from microalgal origin − and thus, a planktonic source has been suspected from the outset. In addition, due to their polyether structural features, a dinophyte origin of AZAs has *ab initio* been suspected. Subsequently, it did not came as a surprise that it was a dinophycean species, *Protoperidinium crassipes*, which was first claimed to be the source of AZAs (James *et al.* 2003). However, production of AZAs by *P. crassipes* could not be verified since then (Gribble 2006). Moreover, in contrast to other identified producers of phycotoxins, which are all primarily phototrophic, *P. crassipes* is a large (> 50 µm) heterotrophic species, known to prey upon other dinophytes (Gribble and Anderson 2006).

During a research cruise with RV Poseidon in the North Sea in 2007, this issue became quite evident when toxin analysis of fractionated plankton samples clearly showed that (1) high amounts of AZAs were found at stations where *P. crassipes* was absent, (2) AZAs could be found in isolated cells of the predatory ciliate *Favella ehrenbergii*, and (3) in

Tab. 1: Currently described species of *Azadinium* (and *Amphidoma languida*). Scale bar = 5 µm



Feature	*Azadinium spinosum* [a]	*Azadinium obesum* [b]	*Azadinium poporum* [c]	*Azadinium caudatum* [d]		*Azadinium polongum* [e]	*Amphidoma languida* [f]
				var. *margalefii*	var. *caudatum*		
Size (length x width)	13.8 x 8.8	15.3 x 11.7	13.0 x 9.8	31.3 x 22.4 [1]	41.7 x 28.7 [1]	13.0 x 9.7	13.9 x 11.9
Length/width ratio	1.6	1.3	1.3	1.2 [2]	1.2 [2]	1.3	1.2
Stalked pyrenoid(s)	1, central episome	no	several (up to four)	no	no	no	1, central episome
Apical and intercalary plates	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	4 apicals, 3 intercalaries	6 apicals, 0 intercalaries
Antapical projection	Small spine	no	no	short horn, long spine	long horn, short spine	small spine	antapical pore
Location "ventral" pore	left side of 1´ (median position)	left side of 1´ (median position)	left side of pore plate	right side of pore plate	right side of 1´ (post. pos.)	left side, suture of 1´and 1´´	right side of 1´ (anterior position)
Shape of pore plate	round /ellipsoid	round /ellipsoid	round /ellipsoid	round /ellipsoid	round /ellipsoid	distinctly elongated	round /ellipsoid
Contact of ventral precing. with intercal.	1´´ in contact with 1a	no	1´´ in contact with 1a	1´´ in cont. with 1a, 6´´ in contact with 3a	1´´ in contact with 1a, 6´´ in contact with 3a	1´´ in contact with 1a	not appl. (no intercalaries)
Shape of plate 4´´	size as other precing., in contact with 3a	size as other precing., in contact with 3a	size as other precing., in contact with 3a	distinctly smaller, no contact with 3a	distinctly smaller, no contact with 3a	size as other precing., in contact with 3a	size as other precingular

[1] size without antapical projection [2] length/width ratio without antapical projection. precing. = precingular plate
References: [a] *A. spinosum* Elbrächter et Tillmann, Tillmann *et al.* 2009. [b] *A. obesum* Tillmann et Elbrächter, Tillmann *et al.* 2010. [c] *A. poporum* Tillmann et Elbrächter, Tillmann *et al.* 2011. [d] *A. caudatum* (Halldal) Nézan et Chomerat, Nézan *et al.* 2012. [e] *A. polongum* Tillmann, Tillmann *et al.* 2012b. [f] *Amphidoma languida* Tillmann, Salas et Elbrächter, Tillmann *et al.* 2012a.

fractionated plankton samples, the largest AZA amounts were found in the small size (<20 µm) class (Krock *et al.* 2009). All these indications led to the isolation of a small dinophyte, which was shown to produce AZA-1 and -2 in axenic culture (Krock *et al.* 2009) and which was identified as the a species, *Azadinium spinosum,* in a newly erected genus (Tillmann *et al.* 2009). Since then, knowledge on the genus has increased considerably. Here, we present an update on the current circumscription of the genus including various aspects on morphological and molecular characterisation, biogeography, and toxin production.

The species

Considering the short period since the first identification of *Azadinium*, the known diversity of the genus has increased rapidly and now comprises five species. *Azadinium spinosum*, the type species of the genus, as well as *Azadinium obesum* were firstly isolated from the same water sample taken from the North Sea off Scotland (Tillmann *et al.* 2009, 2010). Later, *Azadinium poporum* was described from three clones isolated from the southern North Sea off the Danish coast (Tillmann *et al.* 2011). *Azadinium caudatum*, which was initially described in 1953 by Halldal as *Amphidoma caudata,* was recently transferred to the genus *Azadinium* (Nézan *et al.* 2012). Both DNA sequence and morphometric data clearly showed that the species occurred with two distinct varieties, var. *caudatum* and var. *margalefii*. They are easy to distinguish by the different shape of the antapical projection. *Azadinium polongum* − isolated from the Shetland Islands − is the most recently described species of *Azadinium* (Tillmann *et al.* 2012b).

With the description of *Amphidoma languida*, a genus closely related to *Azadinium* could be identified (Tillmann *et al.* 2012a). *Amphidoma languida* has been isolated concurrently with the Irish strain of *A. spinosum* from Bantry Bay, Ireland (Tillmann *et al.* 2012a, Salas *et al.* 2011).

Morphological and molecular characterization

With the exception of *A. caudatum*, all species of *Azadinium* and *Amphidoma languida* are of small size and of similar shape (Tab. 1). They are photosynthetically active with rather typical peridinin pigment profiles (e.g., Tillmann *et al.* 2009). They presumably possess a single chloroplast which is parietally arranged. As a result of a distinct starch cup, stalked pyrenoid(s) are visible in light microscopy for a number of species. All species of *Azadinium* consistently show the Kofoidean plate pattern Po, cp, X, 4′, 3a, 6′′, 6C, 5S, 6′′′, 2′′′′. *Amphidoma languida* exhibits the same hypothecal plate pattern but differs in epithecal plates by having six apical plates and no intercalary series. A very characteristic feature of all species is the prominent apical pore complex (APC) composed of a pore (Po) plate with a central round pore covered by a cover plate (cp) and an X-plate with a characteristic three-dimensional structure. The arrangement of the five sulcal plates is very characteristic for all Amphidomataceae, with a large plate Sa invading the epitheca and a peculiar and conservative Ss plate running from the plates C1 to C6. The second antapical plate may bear a small spine (*A. spinosum*, *A. polon-gum*), a distinct horn with a spine (*A. caudatum*), or a prominent antapical pore (*A. languida*). All species of *Azadinium* and *Amphidoma languida* have a conspicuous ventral pore. However, the position of that pore differs between the species (Tab. 1). Other species determining characters, as the presence or absence of stalked pyrenoid(s), or the shape and arrangement of certain epithecal plates, are listed in Table 1.

Morphology, and in particular the plate tabulation with five different rows of plates, undoubtedly classify the genus *Azadinium* as a member of the dinophycean subclass Peridiniphycidae. This subclass is currently subdivided into two orders, the Peridiniales and Gonyaulacales, with a number of differences discussed in detail by Fensome *et al.* (1993). *Azadinium* clearly exhibits morphological characteristics of both of these orders. The hypothecal plate arrangement, and the presence of six precingular, six postcingular, and six cingular plates, suggest a relationship to the Gonyaulacales. Other general features including the mode of cell division, the plate suture and growth band structure, and the presence of a ventral pore in *Azadinium* seem likewise to reveal a relationship to the Gonyaulacales. However, the epithecal plate arrangement with four apical and three symmetric intercalary plates implies an affinity to the Peridiniales. Moreover, the shape and composition of the APC is typical of the Peridiniales. With the description of *Amphidoma languida*, the taxonomic affiliation of *Azadinium* at the family level was recently clarified. *Amphidoma* was found to be closely related to *Azadinium* with such possible morphological synapomorphies as the cingular and hypothecal plate arrangement, the number and arrangement of sulcal plates, and the characteristic APC. *Amphidoma* and *Azadinium* were thus placed in the family Amphidomataceae by Tillmann *et al.* (2012 a). Molecular phylogenies of the Amphidomataceae based on ribosomal RNA sequence data supported the morphological considerations, but were not able to fully resolve the phylogenetic position of the group within the Dinophyceae (Tillmann *et al.* 2012 a, b). Both morphology and molecular phylogeny thus did not allow for a clear order affiliation and leaves *Azadium* and the family Amphidomataceae with an unclear order affiliation.

Fig. 1: Global records of *Azadinium*/*Amphidoma languida*. (1): Tillmann *et al.* 2009 , 2010. (2): Tillmann *et al.* 2011. (3): Salas *et al.* 2011, Tillmann *et al.* 2012a. (4): Tillmann *et al.* 2012b. (5): Tillmann, unpubl. (6) Nezan *et al.* 2012. (7): Margalef *et al.* 1954. (8) Rampi 1969. (9): Adriana Zingone, pers. com. (10): Tillmann & Busch, unpubl. (11) Hernandez-Becerril *et al. 2012*. (12): Akselman & Negri 2012. (13): Checklist Black Sea phytoplankton. (14): Consuelo Carbonel Moore, pers. com. (15): Potvin *et al.* 2012. (16): Gu *et al.* 2013

Global distribution

Although initially described from the North Sea, the genus *Azadinium* probably is distributed worldwide (Fig. 1). In the North Sea, all five described species have been observed (Tillmann *et al.* 2009, 2010, 2011, 2012b). Based on full characterization of local strains in terms of morphology and sequence information (records 1-5, 6, 9, 15, 16) or based on a few records of single specimens detected by scanning plankton samples by light (record 7-8 referring to the large *A. caudatum*, record 10) or electron microscopy (records 11-14), an increasing number

of global records could be added to a distribution map (Fig. 1). Nevertheless, knowledge on the biogeography of the genus currently is rather limited and patchy. As a consequence of an increasing awareness of the genus, however, the availability of FISH and QPCR as species-specific detection methods (Töbe *et al.* 2013, Potvin *et al.* 2013), and due to the increasing use of "next generation" high throughput sequencing with environmental samples, it is expected that our knowledge on the biogeography of the Amphidomataceae will increase rapidly.

Tab. 2: Origin and AZA profile of strains of Amphidomataceae according to Tillmann *et al.* 2012b, Krock *et al.* 2012 and Gu *et al.* 2013.

Species	Strain	Origin	AZA
A. spinosum	3D9	Scotland 2007	AZA-1 AZA-2 AZA-716
	UTHE2	Denmark 2008	
	SM2	Ireland 2009	
	Shet F6	Shetland 2011	
A. obesum	2E10	Scotland 2007	-
A. polongum	Shet B2	Shetland 2011	-
A. caudatum	AC1/2	Scotland 2011	-
A. poporum	UTHD4 UTHC5 UTHC8	Denmark 2008	AZA-846
	HJ2010	Korea 2010	AZA-858
	G25	Bohai Sea 2007	AZA-858 -920, -928
	G42	East China Sea 2011	AZA-2
	G64	East China Sea 2011	AZA-2
	G60	East China Sea 2011	-
	G66	East China Sea 2011	AZA-872
	G68	South China Sea2011	AZA-2
Amphidoma languida	SM1	Ireland 2009	AZA-816, -830

Toxins

Multiple strains of the type species *A. spinosum* from different locations have consistently been found to produce AZA-1, AZA-2, and AZA-716 (Tillmann *et al.* 2012b). In contrast, *A. obesum*, *A. poporum,* and *Amphidoma languida* have initially been described as non-toxigenic, as none of the known AZAs could be found (Tillmann *et al.* 2010, 2011, 2012a). However, we recently detected four new AZAs in a number of different species. Compared to the previously known AZAs, these new analogs are characterized by a missing methyl group at C39 thus forming a characteristic m/z 348 fragment (Krock *et al.* 2012). Thus, it is evident that the species diversity within this group is also reflected by a high chemical diversity (Tab. 2). We know now that AZA production can also be found in the related genus *Amphidoma* (Krock *et al.* 2012). Six different AZA compounds have been found in strains of *A. poporum* (Tab. 2), with a large variability of AZA-profile among different strains (Krock *et al.* 2012, Gu et al. 2013). AZAs were not detected in all cultivated Amphidomataceae (e.g., *A. obesum*, *A. caudatum* var. *margalefii*, and *A. polongum*), but we cannot exclude the presence of yet unknown and thus undetectable AZA-related compounds.

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Proliferation of 5-hydroxymethyl uracil in the genomes of dinoflagellates is synapomorphic to dinokaryon containing species

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Abstract

Since the original description of 5-hydroxymethyluracil in dinoflagellates by Rae in 1973 there has been little further research in other species. Accordingly, the genomic DNA from members of diverse dinoflagellate genera within dinokaryon were examined and found to have between 10 and 60% of the "T" bases substituted by 5-hydroxymethyluracil. Basal dinoflagellates and other sister taxa did not demonstrate this modified base in substantial quantities, including *Oxyrrhis marina*, three species of *Amoebophrya*: *(Amoebophyra* ex*. sanguinem, Amoebophyra ex. instriatum, Amoebophyra ex. K. veneficum) Perkinsus marinus* and *Chromera velia*. The excessive replacement of thymine by 5-hydroxymethyluracil appears to be a prerequisite for the dinokaryon character to be manifested. Thus, a possible mechanism for dinokaryon evolution would be proliferation of 5-hydroxymethyluracil in the chromosomes and displacement of the stericly incompatible nucleosome structure ultimately resulting in the liquid crystal chromosomes of the dinokaryon containing dinoflagellates.

Keywords: 5-hydroxymethyluracil, dinokaryon, chromosome, dinoflagellate, hmdU

Introduction

When Peter Rae set out to describe the ribosomal gene arrangement in the dinoflagellate *Crypthecondium cohnii,* he noticed a large discrepancy between the GC content as determined by buoyant density versus melt curves. He was able to explain this discrepancy when he found that thymidines were being extensively replaced by the modified based 5-hydroxylmethyluracil (hmdU) Fig. 1 (Rae 1973).

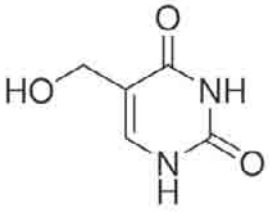

Fig. 1 5-(hydroxymethyl)uracil

This base has the effect of raising the density and lowering the thermal stability of the DNA. He went on to show this replacement was a natural feature of all dinoflagellates he examined. hmdU is also found in trypanosomes where it is further modified to its glucosylated form (Gommers-Ampt *et al.* 1991). This glucosylated form, named base J, occurs in regions of the genome with highly repetitive sequence such as telomeres. Base J functions in trypanosomes by terminating transcription to prevent runoff transcription, thereby allowing transcription to occur in discreet controllable units (Van Leeuwen *et al.* 1997). However, the levels of hmdU, *vis-a-vis* base J, examined so far in trypanosomes are much lower than those recorded in dinoflagellates. Also, the amount of hmdU in non-dinokaryon containing dinoflagellates has not been examined.
The presence and amount of hmdU was quantified in the following experiments using HPLC/MS from taxa throughout the evolutionary lineage of dinoflagellates, and in some cases multiple strains of the same species. These data were used to establish a character state of extensive hmdU replacement; which was then compared to other character states such as condensed and fibroid chromosomes, photosynthetic ability, and parasitic life stages; as well as the overall phylogeny of this family and close outgroups. From the overall dataset it was concluded that a high abundance of hmdU was correlated with condensed fibroid chromosomes, although the exact levels of hmdU varied greatly among and within species and were not correlated to any other characters.

Materials and Methods

Preparation of DNA for compositional analysis:
Approximately 10^6 cells were resuspended in 500µl of 0.1M EDTA pH 8.0, 0.5% SDS which was supplemented with proteinase K to 200µg ml^{-1} and incubated overnight at 55°C. 82.5µl each of 5M NaCl and pre-warmed 10% CTAB, 0.7M NaCl was added and incubated at 55°C for 10 minutes. Samples were extracted with one volume of chloroform for 15 minutes and spun at 10,000 x g for 10 minutes. The supernatant was transferred to a fresh tube and two volumes of Zymo DNA binding buffer was added and mixed by inversion (Zymo Research, Irvine CA). Samples were bound to a Zymo clean and concentrate-5 column for one minute at 10,000 x g and the effluent was discarded. The column was washed twice with ethanol combined washing buffer for one minute at 10,000 x g. 10µl of DEPC treated water was added to the column and incubated at room temperature for five minutes. The unbound DNA was eluted at 10,000 x g for two minutes and quantified using a Nanodrop ND -1000 spectrophotometer. Samples were diluted to 100ng $µl^{-1}$ and 10µl were heat denatured at 95°C for five minutes and snap cooled on ice. 10µl of 40mM MgCl2, 2mM ZnSO4, 1mg ml^{-1} nuclease P1 (>200U) pH adjusted to 6.6 was added and mixed by inversion. Nucleic acids were digested for one hour at 55°C in an MJ thermal cycler with heated lid. 10µl of 2.4U ml^{-1} *E. coli* alkaline phosphatase was added and mixed by inversion. Nucleotides were dephosphorylated for one hour at 37°C. To prepare DNA for analysis of the nucleoside pool, the aqueous phase from an *Amphidinium carterae* preparation following chloroform extraction was centrifuged through an amicon ultra cellulose membrane with a molecular weight cutoff of 3000 to remove incorporated nucleotides. These free bases were then dephosphorylated and analyzed in the same manner as all other samples.

HPLC separation of nucleosides:
Nucleoside composition was determined by LC-UV and LC-MS. Fifteen µl of the digested DNAs were injected onto an Agilent Prep C18 column (LiChrosphere 125 mm x 4 mm, 5 mm bead size RP-18, Agilent; Santa Clara, CA) at 45°C and subjected to a 0.9 ml min^{-1} isocratic elution with 0.1 M triethanolamine acetate pH 6.5 using an Agilent 1100 HPLC, 1100 LC/MS system. UV peaks were detected based on their UV absorbance at 254 and 270 nm. For the MS analysis, the flow from the HPLC (0.9ml min^{-1}) was pumped into the MS electrospray chamber with the addition of 0.1 ml min^{-1} of 1 % formic acid in methanol. The MS was set up for optimal nucleotide/nucleoside ionization by using a fragmentor voltage of 350 V and a capillary voltage of 4000 V. At these settings doubly charged ions were minimized and the total ion abundance of the singly charged parent was at a maximum. Nucleosides standards (adenosine, cytidine, 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyguanosine, 2'-deoxythymidine, guanosine, uridine, 5-methyl deoxycytidine, and 5-hydroxymethyldeoxyuracil) purchased from Sigma (St Louis, MO) were run under the same condition for each sample analysis.

Results

The hmdU quantities expressed as a percent of total "T" bases as well as the GC content and 5-methylC content of the taxa by HPLC/MS is presented in Table 1. The quantity of hmdU ranges from 12.4% in *A. carterae* strain 124 to 66.5% in *Alexandrium tamarense* strain 38-3. Replicate isolates of the same strain varied by 6% while different strains of the same species, *Karlodinium veneficum*, varied by 20%. Relatively high or low quantities of hmdU did not correlate with %GC or genome size and was not related to characters such as parasitic life stage; trophic method; toxin production; pigment composition; or phylogenetic relationship. HmdU was not observed in *Oxyrrhis marina*, two of the three *Amoebophyra* species studied here, *Perkinsus marinus*, or the control species: the raphidophyte *Chattonella marina* or the apicomplexan *Chromera velia*. There were low levels of hmdU detected in the *Amoebophyra* parasite of *Akashiwo sanguineum,* but it is not clear if this is contamination from the host, as part of the sample preparation or incorporation of methylated bases by the parasite. HmdU was detected in the unincorporated nucleotide pool of *A. carterae* at 77% of total "T" bases. This is higher than what was found to be incorporated into the genomic DNA which ranged from 12-47%.
5-methyldeoxycytidine was also detected in many taxa studied here and is also presented in Table 1. Unlike hmdU, the presence of 5-methyldeoxycytidine does not correlate with the presence of a dinokaryon or any other phenotype evaluated here.

Table 1 lists the species name and strain number in parentheses. "%GC" indicates the fraction of guanidine and cytidine of total nucleotides. "%5hmdU" and "%5methylC" expresses 5-hydroxymethyluracil or 5-methylcytidine as a percent of the combined amount of methylated and unmethylated base. Reported errors are calculated from 12 replicate samples and are shown with the average of replicates

Species (strain)	%GC	%5hmdU	%5methylC
A. carterae (CCMP 1314)	55.7 ± 2.2	47.2 ± 6.4	0.0101 ± 0.01
A. carterae (CCMP 124)	52.0	12.46	0.0041
K. veneficum (CCMP 2778)	52.7	43.2	0.0159
K. veneficum (CCMP 1609)	66.9	35.5	0.0312
K. veneficum (CCMP 2936)	53.5	25.6	0.0000
Karenia brevis	54.3	22.5	0.0570
Crypthecodinium cohnii	54.5 ± 3.44	38.7 ± 2.01	0.0033 ± 0.002
Akashiwo sanguineum	56.4	54.0	0.0031
Hematodinium perezi	50.6	13.3	0.0413
Noctiluca scintilans	72.0	48.0	0.0003
Cochlodinium polykrikoides	65.6	27.6	0.0000
Alexandrium tamarense (38-3)	68.3	66.5	0.0028
Alexandrium tamarense (ATSW01-1)	63.3	36.1	0.0054
Amoebophyra ex. A. sanguinea	46.6	5.05	0.0009
Amoebophyra ex. K. veneficum	50.78	0.0	0.0000
Oxhyrris marina	65.5	0.0	0.0094
Chromera velia	51.4	0.0	0.0000
Chatonella marina	38.4	0.0	0.0012

Discussion

These studies confirm previous experiments done in the laboratories of Rae and Borst which showed elevated levels of hmdU in dinoflagellates, but none evident in the apicomplexans. Although hmdU has been shown to exist in trypanosome, the levels are much lower than dinoflagellates and are associated only with highly repetitive regions. In this system thymine is selectively modified to hmdU by either of two proteins, JBP1 and JBP2, that each contain a DNA binding domain and a thymidine dioxygenase domain (Yu *et al.* 2007). Examination of available EST and illumina transcriptomes of dinoflagellates failed to produce any JPB homologues. However a thymidine dioxygenase gene without the DNA binding domain was found, which makes sense since there is hmdU in the nucleotide pools of *A. carterae*, indicating synthesis prior to incorporation prior to incorporation unlike trypanosomes where thymidine is modified to hmdU after incorporation. Thus, although there appears to be convergent evolution between the dinoflagellates and trypanosomes with regards to presence of hmdU and base J, the mode of synthesis is unlikely to be homologous.

However, unique to dinoflagellates is a clear correlation between a proliferation of hmdU in the genome and the presence of the dinokaryon. The most basal members of the dinoflagellates studied here that have high levels of thymidine replacement with hmdU are *Hematodinium perezi* and *Noctiluca scinitlans*, which are the earliest branching dinoflagellates shown to have definitive dinokaryons in at least some life stages. One of the most striking features of the dinokaryon is the lack of histones as the dominant nucleoprotein. This highly basic protein binds to the electronegative phosphate backbone of the DNA strand which wraps around the protein providing tertiary structure. The orientation of the phosphate of hmdU is altered, however, and may be stericly incompatible with histones (Vu *et al.* 1999; Sobell *et al.* 1976). Also, a recent study has

shown that histones, as the major nucleoprotein, have been replaced in dinokaryon containing species by a virally derived nucleoprotein called dinoflagellate/ viral nucleoprotein (DVNP) (Gornik *et al.* 2012). Thus, it is proposed here that the dinokaryon is a result of the acquisition of a DVNP which allowed for the proliferation of hmdU in the chromosomes and a rejection of histone binding, thereby altering the three dimensional conformation of the chromosome to a liquid crystal state. However, it cannot be overstated that dinoflagellates still possess and express histones and that Rae found 10% of DNA in *Crypthocodinium cohnii* to be without hmdU. Therefore, some regions of the chromosomes of dinokaryon containing dinoflagellates probably still retain some traditional chromatin structure.

Acknowledgements:

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Evaluation of LDH-release assay for the detection and analysis of cellular level toxic potential of HAB species

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Abstract

We applied lactate dehydrogenase (LDH)-release assay to estimate toxic potential of harmful phytoplankton through the comparative study between two strains (SUO-1 and FUK) of *Karenia mikimotoi*, in which we used Vero (African green monkey kidney), MJF (yellowtail fin epithelia), and RTgill-W1 (rainbow trout gill) cells as target cells. A live cell suspension of *Karenia mikimotoi* (SUO-1) induced the release of LDH from these cell lines, while the activity of another strain, FUK, was much lower. The cell-free culture supernatants and ruptured cell suspensions of both strains of *K. mikimotoi* were less effective on LDH-release assay. Exposure experiments against abalone and shrimp revealed that SUO-1 showed much stronger lethal effects on these organisms than FUK. Among six phytoplankton species, three species known to be harmful algal species induced the release of LDH to different extents depending on the cell line, whereas the other species, known non-toxic, showed no effects on any cell lines. These results suggest that LDH-release assay is a useful micro-plate assay for estimation of the toxic potential of harmful phytoplankton.

Keywords: LDH-release assay; *Karenia mikimotoi*; HABs; Vero cells; cell culture

Introduction

Harmful algal blooms (HABs) are a serious environmental problem. To determine the toxic mechanisms of HAB species, direct exposure experiments against fish or shellfish have been conducted (Yang *et al.* 1995; Matsuyama *et al.* 1997; Marshall *et al.* 2003; Kim *et al.* 2009). However, such exposure experiments generally require special animal-rearing facilities, mass-culture of phytoplankton, time-consuming procedures, and it is difficult to obtain reproducible quantitative results (Jellett *et al.* 1992; Suzuki *et al.* 1996). To gain insight into the toxic mechanisms and toxic factors in harmful phytoplankton, the use of appropriate cultured cells as an alternative to whole living animals is a promising strategy, and it might be a way to overcome the drawbacks of bioassay described above.

Cytotoxic effects are usually associated with damage to cell membrane integrity, which can be assessed by monitoring the extracellular leakage of intracellular substances that are normally sequestered inside cells. Lactate dehydrogenase (LDH), a cytosolic marker enzyme, is often used to estimate membrane integrity. In fact, the LDH-release assay is a well-known cytotoxicity test in the cell biology field. It is applied to measure the activity of tumor necrosis factor (TNF) (Decker and Lohmann-Matthes 1988), and to analyze cytotoxic lymphocyte-mediated lytic activity against certain target cells (Weidmann *et al.* 1995).

In this study, we verified the usefulness of LDH-release assay as a quick, reliable, and reproducible micro-assay to estimate the toxic potential of harmful phytoplankton. First, we conducted a comparative study between two strains, SUO-1 and FUK, of *Karenia mikimotoi*, in which we used Vero (African green monkey kidney), MJF (yellowtail fin epithelia), and RTgill-W1 (rainbow trout gill) cell lines.

To ascertain whether the results obtained by LDH-release assay reflected the actual toxicity to marine organisms, we carried out exposure experiments on two strains of *K. mikimotoi* against abalone (*Haliotus cracherodii*) and shrimp (*Penaeus semisulcatus*).

To evaluate the LDH-release assay further, we also examined the toxicities of other phytoplankton of various backgrounds to three cell lines by it.

Materials and Methods

Two strains of *K. mikimotoi* were isolated from the Fukuoka Bay (FUK), Japan in 2004 and Suo Nada (SUO-1), Japan in 2006. These clonal strains were kept under the conditions described previously (Zou *et al*. 2010). Vero cells, obtained from the American Type Culture Collection, were cultured as described previously (Oda and Wu 1993). MJF cells established at the National Research Institute of Aquaculture of the Fisheries Research Agency and RTgill-W1 cells obtained from the American Type Culture Collection were cultured at 25°C and 19°C respectively in Leibovitz's L-15 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per mL of medium, penicillin (100 U mL^{-1}), and streptomycin (100 μg mL^{-1}).

LDH activity is measured by a two-step reaction. Target cells maintained in culture flasks were detached from the flasks by treatment with trypsin-EDTA solution in PBS. Detached cells suspended in the growth medium were then seeded into flat-bottom 24-well plates at a concentration of 5 x 10^5 cells mL^{-1} in a final volume of 1 mL $well^{-1}$ and incubated for 24 h. Adherent cells in the 24-well plates were washed once with PBS, and 1 mL of whole-cell suspension of algal cells, the cell-free culture supernatant, or the ruptured cell suspension in each phytoplankton culture medium was added to each well. The plates were incubated for 1h at 26°C under illumination from a fluorescent lamp (200 ± 5 μmol m^{-2} s^{-1}). The supernatant of each well was withdrawn and centrifuged at 2,000 rpm for 5 min. An aliquot (50 μL) of each sample was transferred into a well of a flat-bottom 96-well plate, and then mixed with 50 μL of LDH-detection assay mixture (0.444 mg mL^{-1} of nitro blue tetrazolium, 0.54 mg mL^{-1} of β–NAD^+, 20 U mL^{-1} of diaphorase, and 50 mM lactic acid lithium salt in 10 mM Tris-HCl buffer, pH 8.5). After 30 min of incubation at room temperature, 10 μL of 1 M HCl was added to each well to terminate the reaction. The absorbance of the color that developed, reflecting LDH activity, was determined at 560 nm using a microplate reader (MPR-A4i, Tosoh, Tokyo, Japan). Negative control was determined by spontaneous LDH -release from target cells exposed to phytoplankton culture medium alone, and total cellular LDH content was determined after lysis by ultrasonication of target cells suspended in a phytoplankton culture medium. The levels of LDH released from the target cells into the medium after exposure to phytoplankton cells were expressed as percentages of total celular content, and the value of spontaneous release was subtracted from each value as non-specific release before calculation.

Results and Discussion

As shown in Fig. 1, the *K. mikimotoi* SUO-1 strain induced LDH release from three cell lines to different extents, and the highest levels were observed for the Vero cells. However, the activities of the FUK strain in inducing LDH release from these cell lines were much lower than those of the

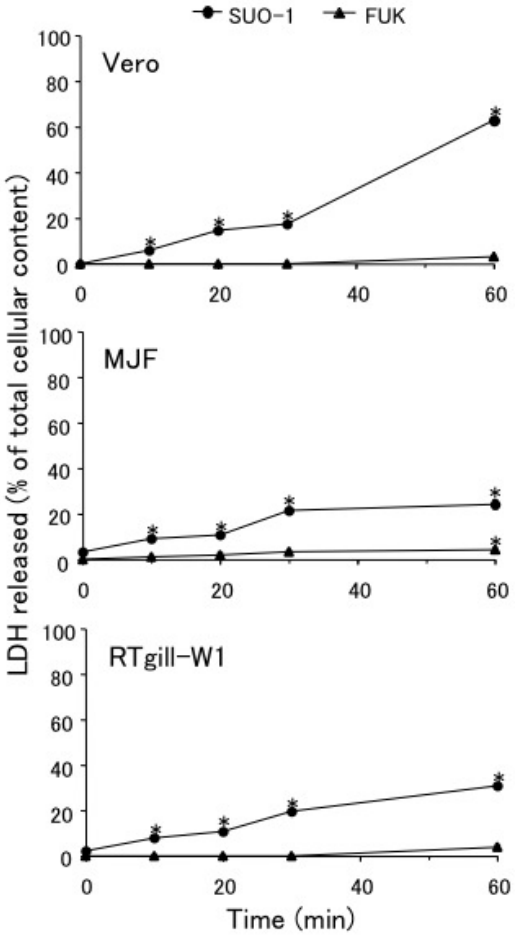

Fig. 1. Time-course analysis of LDH release after exposure to the SUO-1 and FUK strains of *K. mikimotoi* in Vero cells, MJF cells, and RTgill-W1 cells.

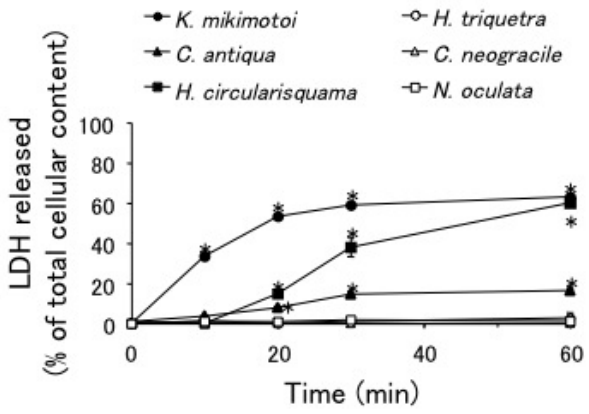

Fig. 2. Time-course analysis of LDH releases after exposure to six species of phytoplankton in Vero cells.

SUO-1 strain. LDH-release from the three cell lines started immediately after exposure to the SUO-1 strain and continued to increase to 60 min. After 60 min of exposure to the SUO-1 strain at a cell density of 4 x 10^4 cells mL^{-1}, more than 60% of the total cellular LDH was released into the medium from the Vero cells, whereas about 20% and 30% of the cellular LDH was released from the MJF cells and the RTgill-W1 cells, respectively. Furthermore, it was found that the activities of the SUO-1 and FUK strains inducing LDH release from Vero, MJF, and RTgill-W1 occurred in a cell-density dependent manner as well, although the activities of the FUK strain were fairly low even at the highest cell density. The activities of the cell-free supernatants and the ruptured cell suspensions of two strains of *K. mikimotoi* inducing LDH-release were much lower than those of the intact cell suspensions. This suggests that the live cell condition is essential for *K. mikimotoi* to induce LDH release from target cells. Probably, certain cytotoxic agents located on the *K. mikimotoi* cell surface make direct contact with the target cell membrane, which can cause membrane damage leading to LDH release. Such toxic substances may not be secreted in free form into the medium during cultivation, and activity may disappear after destruction of membrane integrity or the whole-cell architecture of *K. mikimotoi*.

To ascertain whether the results obtained by the LDH -release assay reflected toxicity with respect to marine organisms, we carried out exposure experiments against abalones (*Haliotus cracherodii*) and shrimps (*Penaeus semisulcatus*). The SUO-1 strain was highly lethal to these organisms, while the FUK strain was almost harmless. 70% of the abalones exposed to the SUO-1 strain died after 72 h, and all the shrimps died after 24 h, whereas no abalones exposed to the FUK strain died after 72 h, and 90% of the shrimps were still alive after 24 h. These results correlate well with the results obtained by LDH-release assay, suggesting that this assay can be used as a simple, small-scale assay to estimate the toxic potential of phytoplankton species.

To verify the applicability of the LDH-release assay to study the toxic effects of various phytoplankton species, five other species of phytoplankton were investigated together with *K. mikimotoi* (the SUO-1 strain). As shown in Fig. 2, *K. mikimotoi* at 1.0 x 10^5 cells mL^{-1} showed the highest LDH-release activities as to Vero cells of the six species tested. *C. antiqua* (8.0 x 10^4 cells mL^{-1}) and *H. circularisquama* (3.5 x 10^5 cells mL^{-1}) also showed the activities in a time-dependent manner. The time-course profiles were somewhat different among the three effective phytoplankton species depending on the target cell line. Especially, immediate LDH-release was observed in the Vero cells exposed to *K. mikimotoi*, whereas there was a lag time before the initiation of LDH-release by the Vero cells exposed to *H. circularisquama* and *C. antiqua*. On the other hand, no significant LDH-releases from any target cells were observed after exposure to *C. neogracile* (2.0 x 10^6 cells mL^{-1}), *N. oculata* (3.5 x 10^6 cells mL^{-1}), or *H. triquetra* (1.6 x 10^5 cells mL^{-1}), which are generally known as harmless algae. These results suggest that the LDH-release assay is useful to distinguish between biologically toxic and less toxic species.

In conclusion, our results suggest that LDH-release assay is applicable for the estimation of toxic potential of harmful phytoplankton species as a simple, quick, and quantitative assay.

Acknowledgements

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Toxin chemistry and toxicity

Application of a Receptor Binding Assay (RBA) to the analyses of PSP toxins in four species of shellfish in El Salvador

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Abstract

The Laboratory of Marine Toxins (LABTOX-UES) has implemented since 2011 a receptor binding assay (RBA) to estimate concentrations of paralytic shellfish poisoning (PSP) toxins in fish products for timely response to toxic outbreaks in El Salvador. Cooperation with the International Atomic Energy Agency (IAEA) and the National Oceanic and Atmospheric Administration (NOAA), has been essential in the implementation of this methodology. El Salvador is the first country in Latin America, after the United States, in applying this test in microplate format. In addition to analyses of toxins in shellfish, the method was adapted to analyze crabs, snails, sea turtles, and other marine organisms which may have acquired PSP toxins through transfers in the food chain. Application of this method allowed for early warning on the occurrence of PSP toxins during the toxic outbreaks in 2010-2011 and 2012 in El Salvador. LABTOX-UES was able to provide timely technical information to state authorities, strengthening their decisions to establish shellfish harvesting closures, mitigating the impact on public health and safeguarding human lives.

Keywords: Red tides, HABs, shellfish toxins, receptor binding assay.

Introduction

Harmful algal blooms of paralytic shellfish poisoning (PSP) toxin producers have a serious impact in public health and cause millionaire losses to the shellfish industry in the Pacific coast of El Salvador (Fig. 1). In 2010, 2011 and 2012 high levels of PSP toxins associated with a bloom of *Pyrodinium bahamense* var. *compressum* were detected in several molluscan shellfish species and led to shellfish harvesting closures. LABTOX-UES, with the support of the International Atomic Energy Agency (IAEA) and the National Oceanic and Atmospheric Administration (NOAA) has implemented a receptor-binding assay (RBA) method for the detection of PSP toxins in shellfish. Following its validation by the AOAC, this method is currently being tested in different laboratories around the world as a fast and sensitive alternative method to the mouse bioassay (MBA).

Material and Methods

Samples of oysters (*Crassostrea iridiscens*), snails (*Plicopurpura columellaris*), crabs (*Carcinus maenas*)

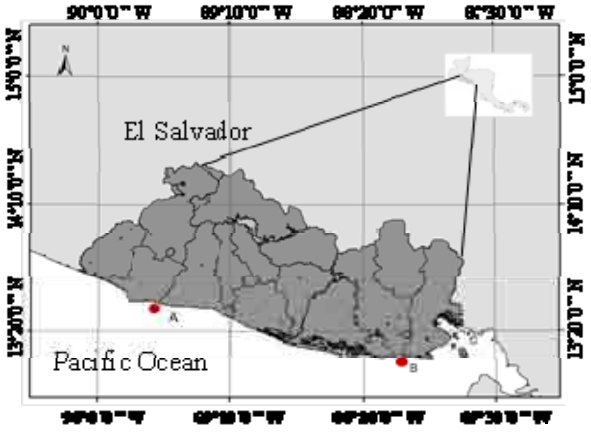

Fig. 1. Location of sampling stations in areas affected by harmful algae events in August and September 2011, in El Salvador. A) Mizata Beach B) El Cuco Beach .

and mussels (*Modiolus capax*) were prepared by homogenizing 1-2 g of flesh that was extracted with an equivalent volume of HCl 0.1N.
The extract, with its pH adjusted to a value of 3-4, was boiled for 5 min, cooled to room temperature, centrifuged (3400 g) and the supernatant collected and filtered (0.45 μm) with a syringe. The acidic

shellfish extracts were analyzed according to the STX receptor binding assay. The method is based on the specific interaction between the toxins and their pharmacological target, i.e. the voltage-gated sodium channel, site 1, for PSP toxins. In this assay, a known amount of radio-labelled saxitoxin ([^{3}H]STX) competes with unllabeled molecules for the sodium channel sites in a rat-brain crude membrane preparation. When the binding equilibrium is reached, free [^{3}H]STX is removed by filtration and the collected receptor-bound [^{3}H]STX is quantified by liquid scintillation counting. The reduction in [^{3}H] STX binding is directly proportional to the amount of unllabeled toxin present (Powell and Doucette 1999).

Results and Discussion

STX-like activity was detected in all samples analyzed. Detection limits of the assay were about 7.6 µg equiv. STX/100 g meat, i.e. 10-fold lower than MBA limits. Samples were diluted, as needed, whenever their concentration of PSP toxins exceeded the linear range of the calibration curve. Similar results were obtained from samples where different dilution factors were used. Concentrations above 2500 µg equiv. STX/100 g meat were detected in oysters and crabs in September 2011 (Fig. 2)
Thus, the RBA can analyze samples with a wide range of toxin levels provided an adequate dilution factor is selected. A standard curve is generated using increasing concentrations of unlabelled toxin

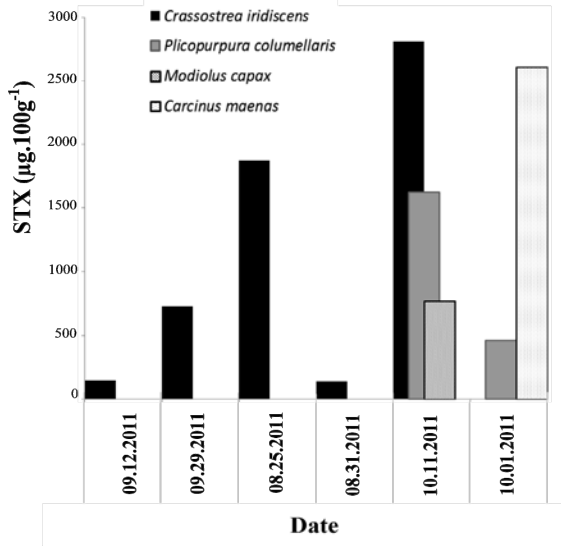

Fig. 2. PSP toxin levels (µg equiv. STX · 100 g^{-1} meat) determined by RBA in 4 (three bivalves and one crustacean) seafood species in El Salvador.

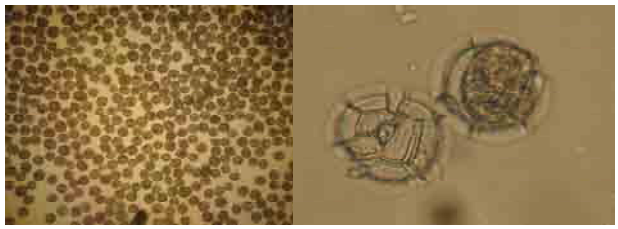

Fig. 3. Micrographs of *Pyrodinium bahamense* var. c*ompressum* from samples collected in El Salvador, summer 2011.

standard; the concentration of toxin in sample extracts is determined with reference to the standard calibration curve (Van Dolah *et al.* 2009). PSP toxins associated with blooms of *P.bahamense* var *compressum*, are recurrent in El Salvador (Fig. 3) (Barraza 2009). LABTOX-UES maintains a monitoring of toxic phytoplankton in El Salvador. Coordination with state institutions is essential. Reports of cell densities and toxins concentration in shellfish are delivered to the National Red Tide committee. Ongoing efforts are being dedicated to implement the RBA method for the analyses of amnesic shellfish poisoning toxins, ciguatoxins and tetradotoxins. High cell densities of *P. bahamense* var *compressum* were found (0, 3, and 12 m) during the sumer 2011 outbreak in El Salvador. Application of the RBA, a functional assay that estimates the toxic potential (toxicity) of the extract in a manner comparable to the MBA has allowed an early warning of the presence of PSP toxins in shellfish in El Salvador and probably contributed to save human lives. It is necessary to implement methods that yield a prompt response to toxic outbreaks and to popularize the RBA method is a challenge for Latin America and the Caribbean countries.

Acknowledgements

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The toxicity of pinnatoxins

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Abstract

Pinnatoxins, members of the cyclic imine group of phycotoxins, are produced by the peridinoid dinoflagellate, *Vulcanodinium rugosum*, and are now known to have a global distribution. The acute toxicities to mice by intraperitoneal (i.p.) injection and by oral administration have been determined for extracts of *V. rugosum* isolates from New Zealand, Australia, Japan, Hawaii and China. Surprisingly, the oral toxicities of several of these extracts were not greatly different from those by injection. The toxicity to mice of purified pinnatoxins E, F and G has also been determined. These substances were all toxic by i.p. injection with LD_{50} values between 12.7 and 57 µg/kg. Pinnatoxin E was much less toxic by oral administration than by i.p. injection, whereas the median lethal doses of pinnatoxin F by gavage and by feeding were only 2 and 4 times higher than those by injection.

Keywords: pinnatoxins, toxicity, *Vulcanodinium rugosum*, Dinophyceae

Introduction

The cyclic imines pinnatoxins A-D were discovered in pen shells, *Pinna* spp., from the South China Sea and Okinawa, Japan, and characterised between 1990 and 1995 (Zheng *et al*. 1990; Uemura *et al.* 1995, Chou *et al*. 1996). Pinnatoxins are fast-acting toxins and potent inhibitors of nicotinic acetylcholine receptors (Hellyer *et al*. 2011).

Pinnatoxins E-G were discovered after mouse bioassay deaths resulted from testing of Pacific oysters and razor clams in South Australia in 2007. Archived Pacific oyster samples from a similar incident in northern New Zealand's Rangaunu Harbour in the 1990s were also found to contain pinnatoxins E and F. This observation led to the discovery of the peridinoid toxin producer (Rhodes *et al*. 2011a). *Vulcanodinium rugosum* (Nézen and Chomérat 2011). *V. rugosum* and/or pinnatoxins in shellfish have now been reported in many parts of the world (Fig. 1).

The toxicites of these compounds have been determined for extracts of *V. rugosum* from Australia, New Zealand, Japan, China and Hawaii (Table 1; Rhodes *et al*. 2011b, Smith *et al*. 2011, Zeng *et al*. 2012) and for the pure toxins (Selwood *et al*. 2010, Munday *et al*. 2012).

Material and Methods

Clonal isolates of *V. rugosum* from New Zealand, Australia, Japan, China and Hawaii were maintained in the Cawthron Institute Culture Collection of Micro-Algae at 25°C in K medium (Keller *et al*. 1987). Batch cultures (5 L) were grown in sterile plastic bags with inserts containing 0.6 M K_2CO_3/ 2.4 M $KHCO_3$ in deionised water (Rhodes *et al*. 2011).

Pinnatoxins E and F were isolated as described previously (Munday *et al*. 2012) from *V. rugosum* cultures. Pinnatoxins were also extracted from *Lyngbya majuscula* collected from Northland, New Zealand (the cyanobacterial mats contained high concentrations of sequestered pinnatoxins), from Pacific oysters (*Crassostrea gigas*), and from sea hares (*Bursatella* sp.). The mats were extracted with methanol, filtered and dried to obtain a residue containing pinnatoxins E and F. The pinnatoxins were separated and purified by liquid-liquid partitioning, flash column chromatography and high-performance liquid chromatography, as described previously (Selwood *et al*. 2010). Pinnatoxin G was isolated from *V. rugosum* strain CAWD183 by sonication with methanol, centrifugation, and further extraction in methanol as described (Munday

Table 1. Toxicity data resulting from the administration of *Vulcanodinium rugosum* extracts containing pinnatoxins to mice and a comparison by different routes of administration

		LD_{50} (mg/kg)				
Isolate	Pinnatoxins present (LC-MS)	i.p injection	Gavage	Voluntary intake	Gavage: i.p. injection	Voluntary intake: gavage
CAWD167 New Zealand	E, F	1.3	2.3	6.0	1.8	2.6
CAWD180 Australia	E, F, G	7.6	22.2	59.3	2.9	2.7
CAWD183 Australia	G (trace F)	2.0	5.5	28.0	2.8	5.1
CAWD188 Japan	G	6.4	19.9	104.0	3.1	5.2
CAWD194 Hawaii	No pinnatoxins	127	NT	NT	NT	NT
CAWD198 China	Unknown compound	12.9	320	NT	24.8	NT

NT: Not tested; i.p.: intraperitoneal

Table 2. Acute toxicity of pure pinnatoxins following administration to mice by intraperitoneal (i.p.) injection, gavage and voluntary intake.

	LD_{50} (mg/kg)		
Pinnatoxin	i.p. injection	Gavage	Voluntary intake
A	37.5	NT	NT
E	57.0	2800.0	NT
F	12.7	25.0	50.0
G	48.0	150.0	400.0
G (metabolite)	25.0	NT	NT
isoE	>100.0	NT	NT

NB: 95% confidence intervals; NT: Not tested. Vehicle for feeding pinnatoxins to mice was cream cheese.

et al. 2012). After centrifugation, the solvent was removed and pinnatoxin G was purified and characterised as described above.

Acute toxicities were determined using female Swiss albino mice, bred at Ruakura. All experiments were approved by the institutional Animal Ethics Committee. Acute toxicities (OECD 2008) of the pinnatoxins were determined as described (Munday *et al*. 2012). The test compounds were dissolved in ethanol, and diluted in 1% Tween 60 in saline for administration by intraperitoneal (i.p.) injection or by gavage. For

voluntary consumption, mice were trained to eat small aliquots of cream cheese (Munday *et al*. 2012). For dosing, an ethanolic solution of the test substance was mixed with the cheese. Solutions were prepared immediately before dosing to avoid hydrolysis of pinnatoxin F.

Results and Discussion

Extracts of *V. rugosum* isolates were tested for their toxicity in mice by i.p. injection and this resulted in the rapid deaths of the mice due to respiratory failure (Table 1). The oral toxicities of several of these extracts were similar to those by i.p. injection, which is not the case for other cyclic imines. For example, gymnodimine and spirolides are 8-23 times less toxic by gavage than by i.p. injection (Munday *et al*. 2012).

When the toxicity ratios for the different administration pathways for the extracts are compared the similar toxicities for the extracts containing pinnatoxin F are highlighted (Table 1).

The toxicities of the pure pinnatoxins have been reported previously (Munday *et al*. 2012) and are summarised in Table 2.

Pinnatoxin F is clearly the most toxic of the pinnatoxins tested. Pinnatoxin E is much less toxic by oral administration than by i.p. injection, possibly due to less or slower absorption due to opening of lactone ring. However, it should be noted that the median lethal dose of pinnatoxin F by gavage and feeding is only 2 and 4 times higher respectively than by i.p. injection.

To date no human illnesses have been confirmed to have been caused by the consumption of pinnatoxin contaminated shellfish (Munday *et al.* 2012).

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Qualitative and quantitative assessment of marine biotoxins in shellfish of the Persian Gulf and Oman Sea

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Abstract

Marine algal toxins produced by harmful microalgae have drawn worldwide attention because of their involvement in human intoxication and socio-economic impacts. Toxins accumulate in the tissues of filter-feeding shellfish exposed to these algae and humans become affected by eating contaminated shellfish. Paralytic Shellfish Poisoning (PSP) and Amnesic Shellfish Poisoning (ASP) are two important kinds of food poisoning caused by shellfish. They are acute and often fatal. In the present study, two groups of toxins, PSP and ASP were analyzed in shellfish from the Persian Gulf (Bandarabbas, Bandarlengeh, Boushehr) and Oman Sea (Chabahar). Sample preparation and extraction were done according to AOAC methods and by ELISA. PSP amounts in shellfish samples ranged from ND-3.962 and ND-1.477 ng/g muscle respectively in these regions. The results showed that toxin contents in shellfish were under regulatory levels in all samples and therefore, seafood was safe for human consumption.

Keywords: marine biotoxins, PSP, ASP, Persian Gulf, Oman Sea

Introduction

Fish is a valuable healthy and widely consumed protein source in the human diet, and it is estimated that 15-20 % of animal protein supply is from aquatic resources (FAO 2008). However, seafood may contain toxins dangerous for the marine fauna and for humans consuming it.

Phytoplanktonic organisms are natural components of the aquatic environments. Under favourable environmental conditions (appropriate light, temperature, oxygen and nutrients) or anthropogenic action (fertilizers input) they may proliferate at a high rate (Hallegraeff, 1993). When particular species dominate the phytoplanktonic community, they may aggregate and form visible dense patches in the water surface commonly known as red tides. These are called harmful algal bloom (HAB) when the proliferating species produce toxins (phycotoxins) that may be a threat to human health or to other parts of the ecosystem. In the sea HABs are mainly caused by dinoflagellates (Batoreu et al., 2005). There are approximately 70 species of dinoflegellates and a smaller number of diatoms, which are currently known to produce phycotoxins (Møestrup et al. 2009). Filter-feeding shellfish concentrate the toxins in their flesh, transfer it through the food web and poisoning occurs when a sufficient quantity of contaminated shellfish is consumed (Garthwaite, 2000). Five major classes of shellfish poisoning have been identified: Neurotoxic Shellfish Poisoning (NSP), Diarrhetic Shellfish Poisoning (DSP), Paralytic Shellfish Poisoning (PSP), Amnesic Shellfish Poisoning (ASP), and Ciguatera Fish Poisoning (CFP). Multiple studies have been undertaken to monitor phycotoxins in the world (Katikou et al., 2009; Wong et al., 2009; Batoreu, et al., 2005), but information on marine biotoxins in shellfish from the Persian Gulf and Oman Sea is still quite limited. The aim of this study was to determine ASP and PSP toxin content in several shellfish species from the northern Persian Gulf (Bandarabbas, Bandarlengeh, Boushehr) and the Oman Sea (Chabahar).

Materials and Methods

Monitoring was carried out at seven stations: Busher, Bandar Lengeh, Bandar abbass and Larak located in the Persian Gulf and Jask and Chahbahar in the Oman Sea. Live mussels were collected from the coast of Bandarabbas, Bandarlenge, Larak and Hormoz Islands, Boushehr, Jask, and Chabahar (Fig. 1). Shellfish specimen were identified and immediately frozen at -20 °C. Identified mussels

were *Callista umbonella* (from Bandarabbas coast), *Circenitha calypiga* (from Bandarlenge coast), *Pinctada radiata* (from Jask coast) and *Chlamys rosenbergii* (from Chabahar coast)

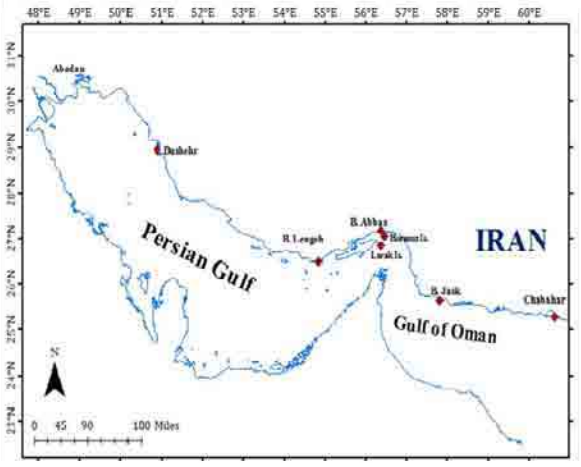

Fig. 1. Sampling locations in the Persian Gulf and Gulf of Oman

The highest PSP and ASP toxicity values measured were 3.961 and 1.477 ng/g respectively in *C. rosenbergii* caught from Chabahar coast, while *Solen* sp. caught from Bandarabbas coast had the lowest toxicity. Statistical analyses showed that there is a significant difference among total PSP and ASP toxicity within different shellfish species.

Conclusions

Most samples collected from the Oman Sea showed positive results for ASP and PSP. Regulatory levels for PSP and ASP toxins in most countries is 0.8 and 20 µg/g mussel meat (FAO, 2004). Our results showed that PSP and ASP toxin levels in all the analyzed shellfish from the Persian Gulf and the ELISA Oman Sea were under regulatory limits and therefore, safe for human consumption .

ELISA kits for PSP and ASP toxins analysis were obtained from the Institute for Marine Bioscience, Canada. The entire testing procedure is described in the manufacturer's manual. Briefly, 50 ml of saxitoxin (STX) standards (included in the kit) or diluted sample extracts obtained during the previous step were allowed to react with the coated antibodies in competition with 50 ml of STX-enzyme complex solution for 60 min at room temperature in the wells of microtiter strips. After a thorough wash of the wells, 50 ml aliquots of substrate and chromogen solution were added and incubation was continued for a further 30 min at room temperature in the dark. The reaction was stopped by adding 100 ml of stop reagent, and OD450 was measured for each well in a UV−Vis spectrophotometer.

Results and Discussion

Total PSP and ASP toxicity in different shellfish species from the Persian Gulf and Oman Sea coasts are shown in Table 1. ASP and PSP toxin levels in *C. umbonella* (caught from Bandarabbas coast), and ASP of *C. calypiga* (from Chabahar coast), *Solen* sp. (from Boushehr coast), and *S. cucullata* (from Hormoz and Larak Island) were under detection levels.

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Table 1. Total PSP and ASP levels among various shellfish from Persian Gulf and Oman Sea

Toxin	Shellfish species					
	Calista Umbonella	*Circenitha calypiga*	*Pinktada radiate*	*Chlamys rosenbergii*	*Solen* Sp.	*Saccostrea cucullata*
PSP	ND	3.070±0.064*	3.544±0.052*	3.961±0.016*	0.273±0.099*	1.015±0.716*
ASP	ND	ND	0.242±0.142*	1.477±0.000	ND	ND

* showed significant differences in toxicity among various shellfish species

Sorting the Fatty Acid Chaff from the Toxin Wheat, or is it All Wheat? - Assigning Dinoflagellate PKS genes to Toxin Synthesis

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Abstract

Success in identifying genes and enzymes that are involved in the biosynthesis of toxins by dinoflagellates has been limited thus far, despite considerable efforts by many groups. The chemical structures of dinoflagellate polyketides suggest that they are produced by modular type I PKS enzymes in some cases with an involvement of a NRPS, for instance in the case of DTX-5a/5b and spirolides. Unfortunately, dinoflagellates also make fatty acids using PKS machinery so it is difficult to discern the machinery involved in toxin synthesis from those involved in fatty acid synthesis. However, we believe there are several research avenues that can be pursued to open the door to this unique biosynthetic machinery. Given the light dependency of karlotoxin production we hypothesize the starter unit for karlotoxin and amphidinol biosynthesis is glycolate that comes from photorespiration. We argue that the acyl carrier protein and acyl transferase (loader) will be different from that used in fatty acid synthesis because the starter unit is different (i.e. glycolate vs acetate). Focusing only on the acyl carrier proteins and acyl transferases we have found two candidate proteins that we believe are involved in the initiation of karlotoxin synthesis.

Keywords: karlotoxin, polyketides, dinoflagellate, biosynthesis

Introduction

The chemical structures of polyketides from dinoflagellates suggest that they are produced by type I polyketide synthases (PKSs), and in some cases with the involvement of a non-ribosomal peptide synthetase (NRPSs). A minimal PKS has an acyltransferase (AT) domain, a β-ketosynthase-(KS) domain, and an acyl carrier protein (ACP). The AT domain covalently transfers a specific carboxylic acid from acyl-CoA to the ACP, which is then condensed by the KS domain to another ACP-bound acyl chain. A PKS module may have optional β-ketoacyl reductase (KR), dehydrogenase (DH), and enoyl reductase (ER) domains, which reduce the β-ketone to an alcohol, dehydrate the alcohol, and saturate the resultant double bond, respectively. In analogy, a minimal NRPS provides an adenylation domain (A), which specifically activates an amino acid, a peptidyl carrier protein (PCP), and a condensation domain (C) that creates a peptide bond between two PCP-bound amino acids. Thioesterase (TE) domains may release, and cyclize the final enzyme products.

Type I PKSs and NRPSs usually consist of large, non-iterative, multidomain enzymes. Modular type I PKSs and NRPSs form megasynthetases that generally follow a colinearity rule, where one module extends a growing acyl or peptidyl chain by one particular unit. Each PKS module carries a set of catalytic domains that perform one round of polyketide elongation and modification. During these processes, an AT domain attaches the corresponding acyl-CoA building block onto an acyl carrier protein domain (ACP), a KS domain elongates the polyketide chain with this acyl unit and optional additional domains further modify the resulting intermediate. An example of this modular synthesis is presented in Fig. 1 for the first 14 carbons of karlotoxin with glycolate being the starter unit rather than acetate. For these 14 carbons, 8 KS and AT domains are required with only 7 KRs and 3 and 2 DHs and ERs modules. However, these 8 modular genes have not been found in any karlotoxin producing species.

The frequently observed close correlation between the domain architecture and the sequence of functional groups in the polyketide chain, codified as the 'colinearity rule', has enabled direct prediction of polyketide structures from genomic sequences and

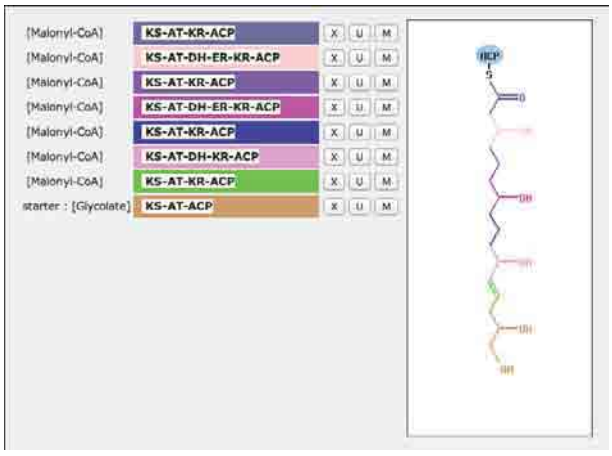

Fig. 1. Predicted PKS modular structure for the first 14 carbons of karlotoxin. The starter unit was glycolate rather than acetate.

vice versa. **However, this colinearity appears to be broken in dinoflagellates.**

Kubota *et al*. (2006) screened genomic DNA from five amphidinolide-producing and eight non-producing dinoflagellate strains by degenerate PCR for the presence of β-ketosynthase (KS)

fourteen unique KS domains were detected. These sequences were exclusively present in amphidinolide producer strains, and a genomic fosmid DNA library was constructed from the amphidinolide-producing strain *Amphidinium* sp. Y-42. Kubota et al. (2006) detected a single clone out of a total of 100,000 PCR-screened clones, which harbored PKS-related sequences, and the entire fosmid insert (36.4 kb) was sequenced. The fosmid insert had six sequence regions, KS, AT, DH, KR, ACP, and TE that were related to type I PKS genes. Their genomic arrangement was unusual however, as several frame-shifts occurred within and between catalytic domains. The protein-coding region was flanked on both sides by long stretches of non-coding sequence, and the mid section of the protein-coding region contained a 4 kb stretch of sequence that presumably represented an intron. Only approximately 15% of the 36.4 kb long fosmid insert consisted of protein-coding sequence. This sequence encoded putative catalytic functions for only a single elongation cycle of a 26-membered polyketide. If one would extrapolate based on these data, all genes required for the production of amphidinolide may occupy up to 500 kb of genomic DNA. Further, it would not be certain, whether they were present on the same locus, or distributed throughout the genome. This study exemplifies the huge challenges associated with characterizing biosynthesis genes in dinoflagellates on the genomic level, regardless of the sequencing technology used. Unfortunately, sequences obtained in the study by Kubota et al. (2006) were not deposited in GenBank, preventing further analysis. Similarly, when Bachvaroff and Place (2008) tried to find other modules of a KR gene in *A. carterae* no other PKS modules were found within 12 kb of genomic DNA. This gene was also interspersed with numerous introns. And lastly, when Monroe and Van Dolah (2008) characterized the PKS cDNAs from *K. brevis* they only found transcripts that contained one catalytic module rather than multiple modules expected for a modular Type I PKS transcript.

Recently a novel group of modular PKSs that diverge from the canonical (*cis-AT*) type architecture have been described (Nguyen 2008). These *trans-AT* PKSs are characterized by the absence of an integrated AT domain in each module which is complemented by free-standing acyl transferases. Phylogenetic data suggest that these *trans*-AT PKSs evolved independently from cis-AT PKSs. *We believe this is the mode of polyketide synthesis in dinoflagellates*. In addition to the unique acyl transfer mechanism, the enzymes are noteworthy for exhibiting highly aberrant architectures with modules carrying novel catalytic domains or domain orders, or having no apparent function or relation to polyketide structure. These could only be compared using bioinformatic approaches of the amino acid sequences.

Approach

Given the light dependency of karlotoxin production we hypothesize the starter unit for karlotoxin and amphidinol biosynthesis is glycolate that comes from photorespiration. A previous study by Murata's group established the polyketide origin of three amphidinols, AM2, AM3 and AM4 (Houdai *et al*. 2001) using ^{13}C labeled acetate. The enrichment patterns observed revealed that the carbon chain of AM17 is derived entirely from acetate units, including the pendant carbon atoms. Both C-1 and C-2 of the chain were however, unlabeled, similar to a result obtained for other amphidinols, and this portion of the chain was inferred to result from a glycolate starter unit as observed in okadaic acid and its analogs (Wright *et al*. 1996). Recently, glycolate was shown to be the starter unit for yessotoxin

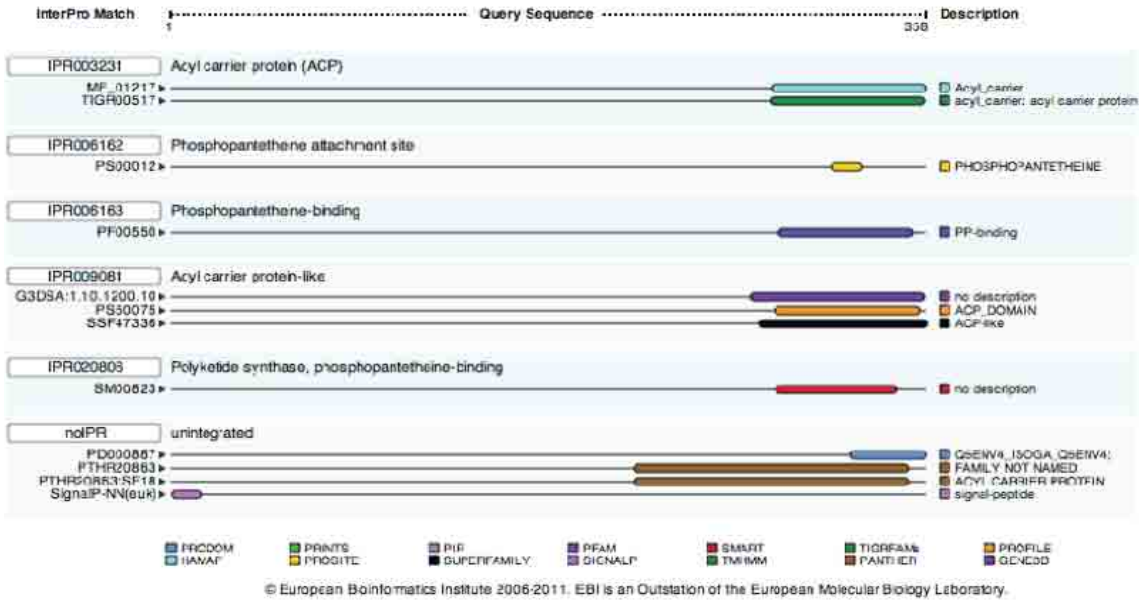

Fig. 2. Predicted domains for a plastid acyl carrier protein cDNA from *K. veneficum* (CCMP 2778)

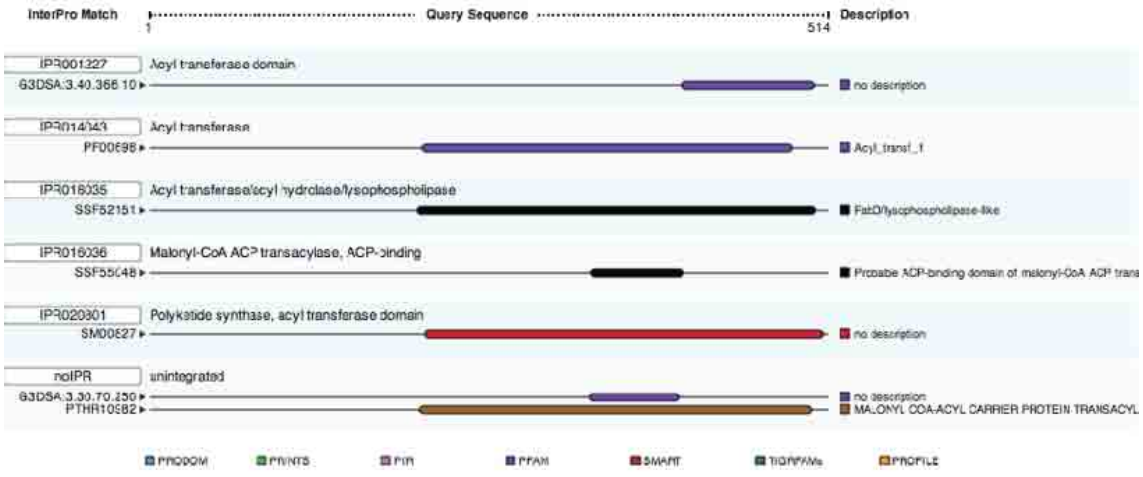

Fig. 3. Predicted domains for a CA-Acyl Carrier Protein Transacylase cDNA from *K. veneficum* (CCMP 2778).

synthesis (Yamazaki *et al*., 2010) and adding glycolate to the culture media enhances gymnodimine production (Mountfort *et al*., 2006). This is currently being tested with feeding studies on *K. veneficum* and *A. carterae*.

From the current annotation of the full-length cDNA libraries (>160,000) of *K. veneficum* and *A. carterae* (NSF Microbial Genome Sequencing Program grant #EF-0626678, "Dinoflagellate full-length cDNA sequencing") we have already identified greater than 200 genes potentially involved in fatty acid and karlotoxin (or amphidinol) synthesis. To sort through these candidate genes we have focused on the initiation of the PKS cycle looking for enzymes that might transfer glycolate to an acyl carrier protein and could also accommodate a large growing polyketide chain. We have found two candidate proteins that we believe are involved in the initiation of karlotoxin synthesis. The first (Fig. 2) is a large acyl carrier protein (ACP) (359 amino acids) that differs significantly from the smaller ACPs (~134 amino acids). This protein has a chloroplast targeting sequence. We envision the numerous smaller cytoplasmic ACPs are involved in fatty acid synthesis while the larger plastid protein receives the starter glycolate for karlotoxin synthesis. The second candidate is a malonyl carrier protein transacylase (Fig. 3) that is phylogenetically

distinct from the other CoA-acyl carrier protein transacylases found in the libraries. We envision this transacylase charges the ACP with glycolate in the chloroplast. We are currently testing these hypotheses using antibodies to each of these proteins.

Conclusion

Only by focusing on the initiation of the PKS cycle can we sort the metabolic players involved in toxin biosynthesis from those involved in fatty acid synthesis. Even if this approach proves successful it is highly likely that many of the same players may be involved in both processes.

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Cyanophyceae and freshwater algal blooms

Environmental factors that influence freshwater cyanobacterial populations in the Piedmont region of North Carolina, USA.

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Abstract

In shallow surface waters, cyanobacterial blooms can promote light attenuation, hypoxia following bloom die-back, and taste and odor concerns in municipal water supplies. In some regions, dominant species may also produce cyanotoxins that can both alter ecological functions in aquatic systems and pose human health risks. Therefore, the purpose of this study was to characterize how environmental physiochemical factors promote cyanobacterial blooms in shallow freshwater systems of the Piedmont physiographic region of North Carolina, USA. In this study, we focused on four dominant cyanobacterial genera (*Anabaena* spp., *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii*, and *Microcystis* spp.) that occur in Piedmont rivers, reservoirs, and impoundments. We used multivariate analyses on environmental parameters and cyanobacterial populations sampled over a 12 year period (2000 to 2011). The results suggest that cyanobacteria were strongly influenced by water-column phosphorus (when considering populations on a unit mL^{-1} basis) and total Kjeldahl nitrogen (on biovolume basis). Moreover, *Anabaena* and *Aphanizomenon* populations were higher during warmer periods, and along with *Microcystis*, were more abundant when phosphorus levels were elevated. In contrast, *Cylindrospermopsis* concentrations were more abundant when nitrogen levels were comparatively high.

Keywords: *Anabaena*, *Aphanizomenon*, Cyanobacteria, *Cylindrospermopsis*, *Microcystis*, Water Quality.

Introduction

In North Carolina, nonpoint source runoff has been identified as a major source of ecosystem disturbance in surface waters (Lenat and Crawford 1994). Industrial, urban, and agricultural expansions have been attributed to accelerated eutrophication and declines in water quality that promote changes in phytoplankton community composition (Anderson *et al*. 2002; Piehler *et al*. 2004). Occurrences in harmful algal blooms have increased in North Carolina over the last few decades, and are a growing concern as their toxins (including cyanotoxins) may adversely impact humans, livestock, and aquatic food webs (Skulberg *et al*. 1998; Chorus and Bartram 1999; Touchette *et al.* 2007). The purpose of this study, therefore, was to determine what environmental water quality factors influence the production and maintanence of four dominant cyanobacterial genera (*Anabaena* spp., *Aphanizomenon flos-aquae*, *Cylindrospermopsis raciborskii*, and *Microcystis* spp.) in freshwater systems of the North Carolina's Piedmont region.

Material & Methods

This study was conducted as part of a routine monitoring program that collected physiochemical and biological data from surface waters between January 2000 and December 2011 (from 47 different locations involving 29 North Carolina counties). Water quality sampling was conducted either monthly (in some locations) or during episodic bloom events, and included subsurface physiochemical measurements of temperature, pH, dissolved oxygen (DO), and conductivity using a Hydrolab Surveyer-4 (Hydrolab Corp., Austin, Texas, USA). In some cases, increased monitoring frequencies occurred during periods of high cyanobacterial densities or bloom events. During such events, ambient nutrient conditions were also evaluated including, inorganic nitrogen (NO_x^- and NH_4^+), total Kjeldahl nitrogen (TKN), total suspended solids (TSS), and total phosphorus (TP). Additionally, phytoplankton samples (preserved in the field with Lugol's solution and stored on ice) were collected within the photic zone (defined here as

twice the Secchi depth). Cyanobacterial abundances were numerated using an Utermöhl settling chamber (Phycotech Inc., St. Joseph, MI, USA) magnified at 300X on an inverted microscope (Leitz Diavert).

Data analyses included a high number of field-based observations over the 12-year period, including 133 for *Anabaena* spp., 113 for *Aphanizomenon flos-aquae*, 232 for *Cylindrospermopsis raciborskii*, and 98 for *Microcystis* spp. (Table 1). Multivariate canonical correspondence analyses (CAA) were performed on data using PC-ORD statistical software (version 4; MJM Software Design, Gleneden Beach, Oregon, USA). Cyanobacterial abundances (both units mL^{-1} and biovolume) were compared against environmental physiochemical variables, and the resultant factors after 100 randomizations were tested for significance using a Monte Carlo permutation test ($p < 0.05$). Data was also grouped between high and low harmful algal abundances (high was arbitrarily defined as a biovolume > 500 µm3 mL^{-1}) and statistical comparisons between environmental parameters at high and low algal abundances (including absence of designated species) were conducted using Kruskal-Wallis one-way ANOVAs.

Results & Discussion

The observed cyanobacterial populations maintained a strong seasonal component wherein most bloom events (300 out of 323) occurred in mid- to late-summer and continued through early autumn. Indeed, *Anabaena* as measured through biovolume were significantly influenced by high temperatures (most abundant at ~28.5°C; p=0.003), as well as high TP, TKN, and TSS ($p<0.05$; Table 2). Similarly, *Aphanizomenon* was influenced by high temperatures (most abundant at ~28.8 °C; p=0.002), but also during lower TKN and/or TSS ($p<0.05$). In contrast, *Cylindrospermopsis* was found to be more abundant during low inorganic N (both NH_4^+, and NO_x^-) and high organic N (i.e., TKN) conditions ($p< 0.05$). Notwithstanding, *Microcystis* was principally influenced by organic phosphorus levels (Table 2; p=0.013).

CAA revealed significant patterns between environmental physiochemical parameters and cyanobacterial abundances for the first three components ($p<0.01$, Monte Carlo test results). The first two factors were able to account for 23.2 and 25.8 percent of the total variance for units and biovolume, respectively. Eigenvalues were higher for the first axis (0.237 and 0.334, compared to 0.067 and 0.213 for axis-2), and therefore interpretation of Figure 1 should focus largely on horizontal components. Overall, the strongest coefficients (> 0.20) for the first two components were TP, NH_4^+, TKN, and TSS for unit enumerations, and TKN and NO_x^- for biovolume. For axis-2 the dominant environmental parameters were NH_4^+ and NO_x^- for units evaluations NO_x^- and TKN for biovolume (Table 3).

Overall, the data reveal the important role of nutrients in bloom development and maintenance as phosphorus levels were greater during higher abundances of *Anabaena* and *Microcystis*. Furthermore, both organic- and inorganic-N were higher during periods of high abundances of *Anabaena*, *Aphanizomenon*, and/or *Cylindrospermopsis*.

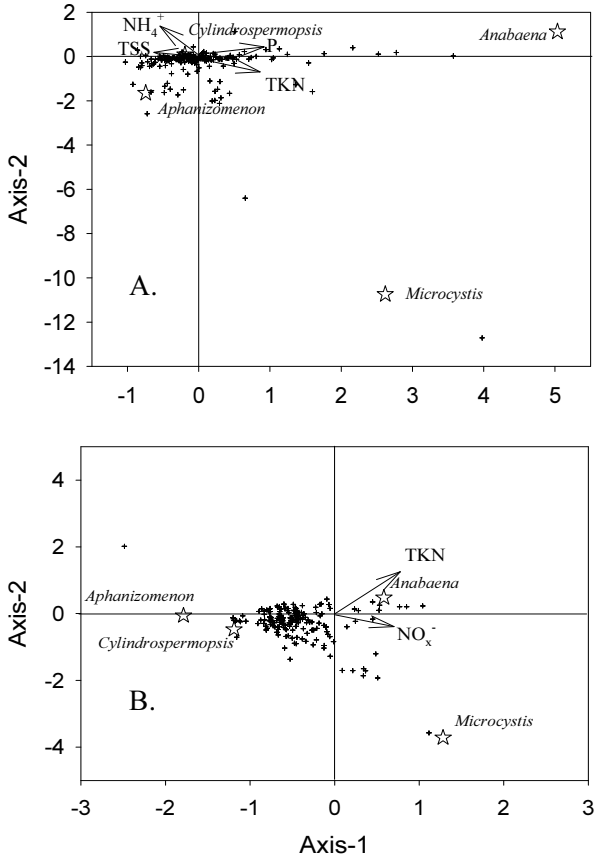

Fig. 1. CCA defined ordination of environmental samples within space. The strongest environmental variables ($r^2 > 0.20$ with any axis) are presented as vectors in the bi-plot overlay. The ordinations include cyanobacteria enumerated as units mL^{-1} (upper panel A) and biovolume (lower panel B).

Table 1. List of cyanobacterial species included in this study. Data presented includes number of observations (Num. Obs.) for each taxonomic group, mean density (± 1 S.E; for both biovolume and units), maximum density (Max.), and minimum density (Min.).

Species	Num. Obs.	Density	Max.	Min.
Biovolume (μm^3 mL^{-1})				
Anabaena	113	2,566 ± 688	5,246	1
Aphanizomenon	113	456 ± 75	5,044	1
Cylindrospermopsis	232	310 ± 38	5,246	1
Microcystis	98	248 ± 83v	2,586	1
Units (mL^{-1})				
Anabaena	113	835 ± 207	28,465	1
Aphanizomenon	113	1,104 ± 181	20,574	1
Cylindrospermopsis	232	25,900 ± 3,200	487,900	1
Microcystis	98	309 ± 59	7,552	1

Table 2. Abiotic parameters collected from Piedmount freshwater systems grouped between high and low algal abundances, where high abundance was arbitrarily selected as greater than 500 um^3 mL^{-1} (biovolume). Parameters include pH, temperature (°C), total phosphorus (TP; mg L^{-1}), total Kjeldahl nitrogen (TKN; mg L^{-1}), ammonium (mg L^{-1}), nitrate + nitrite (NO_x^-; mg L^{-1}), and total suspended solids (mg L^{-1}). Statistical differences were based on a nonparametric Kruskal-Wallis one-way ANOVA, where $p < 0.05$ are indicated by an asterisks.

BioVol (genera)	pH	Temp	TP	TKN	NH_4^+	NO_3^-	TSS
Anabaena (μm^3 mL^{-1})							
Low (<500)	7.7±0.1	25.9±0.4	0.06±0.01	0.66±0.03	0.18±0.15	0.09 ± 0.03	7.0 ±0.3
High (>500)	7.9±0.2	28.5±0.6*	0.09±0.01*	1.08±0.10*	0.03±0.01	0.01±0.01	9.7±0.9*
Cylindrospermopsis							
Low (<500)	8.5±0.9	26.1±2.0	0.07±0.01	0.72±0.05	0.18±0.01	0.09±0.01	7.4±0.6
High (>500)	8.4±1.8*	27.9±5.8	0.05±0.01	0.80±0.16*	0.01±0.01*	0.01±0.01*	7.8±1.6
Microcystis							
Low (<500)	8.5±0.6	26.4±0.2	0.07±0.01	0.72±0.05	0.16±0.01	0.08±0.01	7.4±0.6
High (>500)	8.4±3.8	25.5±11	0.13±0.06*	1.10±0.49	0.01±0.01	0.25±0.11	9.8±4.4
Aphanizomenon							
Low (<500)	8.6±0.7	26.1±2.0	0.07±0.01	0.74±0.06	0.17±0.01	0.09±0.01	7.7±0.5
High (>500)	7.7±1.8	28.8±6.8*	0.05±0.01	0.72±0.17*	0.02±0.01	0.01±0.01*	4.8±1.1*

Table 3. Multiple regression canonical coefficients from CAA between cyanobacteria density (Units mL^{-1} and biovolume) and environmental parameters.

Density (Parameter)	Axis 1	Axis 2	Axis 3
Units (mL^{-1})			
pH	0.008	0.013	0.034
Temperature	-0.068	0.016	0.068
Conductivity	-0.005	0.007	-0.012
Phosphorus	0..501	-0.168	0.142
TKN	0.222*	0.135	-0.115
Ammonium	-0.251*	0.637*	0.052
Nitrate/Nitrite	-0.112	-0.563*	-0.146
Suspended Solids	-0.243*	0.072	-0.128
Biovolume (μm^3 mL^{-1})			
pH	-0.014	0.080	-0.042
Temperature	-0.025	0.112	-0.044
Conductivity	0.069	0.008	0.041
Phosphorus	-0.059	-0.128	-0.613*
TKN	0.615*	0.291*	0.380*
Ammonium	-0.073	0.132	0.062
Nitrate/Nitrite	0.221*	-0.327*	0.066
Suspended Solids	0.023	-0.180	0.303

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Microcystin-encoding gene cluster in *Synechococcus* strain isolated from Great Mazurian Lakes

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Abstract

We tested 43 picoplanktonic strains of *Synechococcus* isolated from the Great Mazurian Lakes for the presence of the microcystin-encoding gene cluster (*mcy*), responsible for hepatotoxins, microcystins production. In one of the analyzed strains, BE0807B, the presence of three marker genes, necessary for microcystins production, *mcy*A, *mcy*D and *mcy*E, was revealed. The sequencing of the *mcy* genes from this uniclonal culture showed that they were highly similar to *Planktothrix agardhii mcy* genes. We found similar *mcy* genes in environmental samples in the picocyanobacterial size fraction (0.2-2.0 μm), and in the *Synechococcus* strain BE0807H, though the DGGE analysis revealed presence of *Planktothrix* 16S rDNA-ITS in these samples. Although the ability of picocyanobacteria to produce microcystins has already been confirmed from environmental and laboratory data, it is the first time that the actual sequences of the *mcy* gene cluster were identified in picocyanobacteria strains. The results suggest toxicity potential in the communities of picocyanobacteria, though it needs further studies.

Keywords: Picocyanobacteria, *Synechococcus*, DGGE, *mcy* gene cluster, *Planktothrix*

Introduction

Picoplanktonic cyanobacteria discovered in late 70s of the previous century are ubiquitous aquatic microorganisms. In freshwaters *Synechococcus* and *Cyanobium* represent them, while in the oceans, *Prochlorococcus* and *Synechococcus* (Partensky *et al.* 1999, Callieri 2007). Picocyanobacteria occur in the natural environment in varying numbers and depending on the environmental conditions can represent a significant percentage of the total number and biomass of phytoplankton in freshwater and in marine environment (Agawin *et al*. 2000). Although picocyanobacteria are generally considered nontoxic, there are reports of the ability of picocyanobacteria to produce hepatotoxins from the microcystins group (Oudra *et al*. 2002, Carmichael and Li 2006). However so far no actual sequences of the *mcy* gene cluster had been identified in picocyanobacteria strains. The aim of this study was to i) search for presence of the *mcy* gene cluster in picocyanobacteria strains isolated from the Great Mazurian Lakes in Poland, ii) sequence the potential *mcy* genes from cultured strains and iii) verify the occurrence of picocyanobacterial *mcy* genes in environmental samples.

Methods

Cultures and environmental samples

Forty three (PC-rich and PE-rich) *Synechococcus* strains isolated from the Great Mazurian Lakes located in northeastern Poland (Jasser *et al*. 2010) were cultured in BG11 medium in 14:10 L:D cycle at 16 μE m^2 s^{-1} cool light irradiance at temperature 18°C. For environmental samples phytoplankton size fraction of 0.2-2.0 μm, characteristic for picocyanobacteria, was collected from eight Mazurian Lakes in April 2012.

Analyses

DNA from 43 strains was isolated using a commercial GeneMATRIX Bacterial Genomic Gram $^{+/-}$ DNA Purification Kit (EURx). Three *mcy* genes (*mcy*A, *mcy*D and *mcy*E) were amplified with previously described primers (Hisbergues *et al.* 2003; Rantala *et al.* 2004). Sequences of obtained *mcy* genes were compared using a BLAST tool with three most

frequent microcystins producers (*Microcystis*, *Planktothrix* and *Anabaena*). To verify if the picocyanobacteria, which contain the *mcy* gene cluster occur in the Mazurian Lakes, picophytoplankton fraction encompassing picocyanobacteria was separated by filtration through 2.0 µm membrane filters and then deposited on 0.2 µm polycarbonate membrane filters. DNA was isolated using GeneMATRIX Soil DNA Purification Kit (EURx) and the presence of toxicity genes was checked. To verify the presence of other cyanobacteria taxa in the samples, fragments of each filter were examined under epifluorescent microscope. To ensure that the picocyanobacteria strains and prefiltered environmental samples from one of the lakes, used for further analyses, were not contaminated with other DNA, internal transcribed spacer (ITS) of 16S rRNA was amplified with CSIF and 373R primers (Janse *et al.* 2003; Wilmotte *et al.* 1993). Then the denaturing gradient gel electrophoresis (DGGE) of amplified ITS was performed. Bands obtained in the analysis were cut from gel and sequenced.

Results

All three tested markers (*mcy*A, *mcy*D and *mcy*E) were detected in two strains isolated from eutrophic Lake Bełdany – PE-rich BE0807B and PC-rich BE0807H (Fig 1).

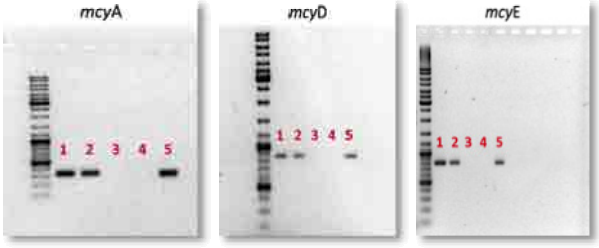

Fig 1. The presence of tested markers in strains BE0807B (band 1), BE0807H (band 2), in closely related strains (bands 3 and 4) and in reference strain of *Microcystis* (band 5).

The three markers were also found in at least one of the samples (0.2-2.0 µm size fraction) from eight studied lakes (Fig 2).
No cyanobacteria other than *Synechococcus* were detected on the 0.2 µm pore size filters after pre-filtration, nor earlier in the cultures of pico-cyanobacteria. The DGGE analysis of 16S rRNA-ITS performed for the DNA isolated from the two cultures

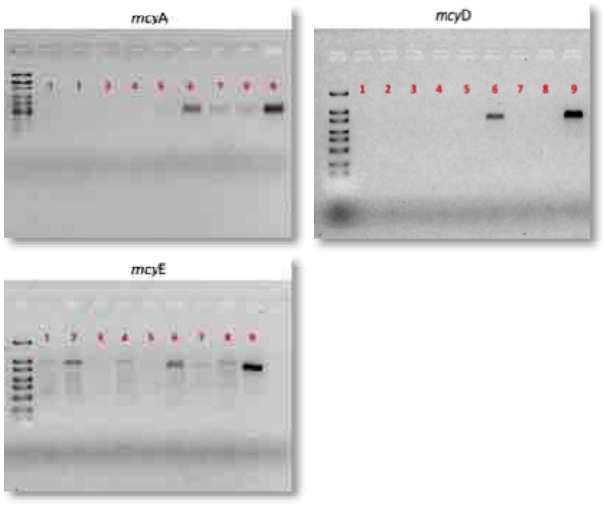

Fig 2. The presence of three *mcy*-genes fragments in picophytoplankton fraction from eight Mazurian Lakes: 1 – Niegocin, 2 – Jagodne, 3 – Szymon, 4 – Tałtowisko, 5 – Tałty, 6 – Mikołajskie, 7 – Śniardwy, 8 – Bełdany, 9 – reference sample.

revealed only one band for the PE-rich BE0807B strain, confirming that this strain was not contaminated with any other DNA. The ITS sequence of this strain showed the highest similarity (99%) with *Synechococcus* strains (LM94 and BO9404) from clade B (Crosbie *et al*. 2003). Also earlier analyses of *cpc*BA-IGS assigned this strain to group B (Jasser *et al*. 2011). We have not detected the ITS of the BE0807B strain in our environmental samples. On the other hand, the BE0807H strain and the prefiltered environmental samples from the studied lake revealed presence of several bands in 16S rRNA-ITS DGGE analysis, including Planktothrix ITS.

The three *mcy* genes obtained from the DNA isolated from *Synechococcus* BE0807B strain showed the highest similarity (97-99%) to the genus *Planktothrix* and between 70 and 80% with the other two common microcystins producers; *Microcystis* and *Anabaena* (Tab 1). The *mcy*A gene sequence similarity with the *Planktothrix agardhii* CCNP1304, which DNA was used as reference sample, was also as high as 95%.
Results obtained in this study indicate the existence of potentially toxic freshwater picocyanobacteria. Earlier results concerning picocyanobacteria have not yet provided sequences of genes encoding microcystins, though the ELISA and HPLC analyses confirmed the presence of microcystins in

Table 1. Comparison of the *mcy*A,D,E gene sequences from BE0807B strain with sequences from the most frequent microcystins producers.

	Organism	GenBank Accession number	Sequence similarity
***mcy*A**	*Microcystis aeruginosa* NIES-843	NC_010296.1	70%
	P*lanktothrix agardhii* NIVA-CYA 12618	AJ441056.1	99%
	A*nabaena* sp. 90	AY212249.1	72%
***mcy*D**	*Microcystis aeruginosa* NIES-843	NC_010296.1	81%
	P*lanktothrix agardhii* NIVA-CYA 12618	AJ441056.1	97%
	A*nabaena* sp. 90	AY212249.1	81%
***mcy*E**	*Microcystis aeruginosa* NIES-843	NC_010296.1	81%
	lanktothrix agardhii NIVA-CYA 12618	AJ441056.1	99%
	nabaena sp. 90	AY212249.1	80%

environmental samples and isolated strains of *Synechcoccus* (Carmichael and Li 2006) and of *Synechocystis* (Oudra *et al*. 2002). Our results provide such sequences, though the similarity of *Synechococcus mcy* sequences to *Planktothrix* found in our study is puzzling. This similarity may initially suggest contamination of the samples by non-*Synechococcus* DNA, but that was ruled out, both by microscopic and molecular analyses. Thus it seems that either the *mcy* genes in *Synechococcus* are closely related to *Planktothrix*, which is unlikely when compared with the 16S phylogeny (Rantala *et al*. 2004) or that they could be obtained via lateral transfer. The horizontal gene transfer as a way to pass on the ability of toxin production was suggested to explain i.e. the patchy distribution of *mcy* genes among cyanobacterial taxa (Nakasugi *et al*. 2007).

However other facts, such as congruency between housekeeping and *mcy* encoding genes as well as *mcy* remnants in strains, which do not produce microcystins imply rather a vertical inheritance of these genes (Rantala *et al*. 2004, Christiansen *et al*. 2008). Thus further studies are needed to shed the light on the genetic bases of *Synechococcus* potential of hepatotoxins production.

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Control of *Microcystis aeruginosa* bloom by using microorganisms in waters from reed community and water plant bed

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Abstract

There is an urgent need of strategies for preventing and/or reducing negative impacts of toxic cyanobacterial blooms. Algicidal bacteria are promising agents against cyanobacterial blooms. Experimental studies on control of *Microcystis aeruginosa* blooms were conducted using microorganisms in waters of a reed community and water plant zone in Lake Ohnuma district, Japan. Cyanobacterial bloom samples were collected at a harbor of Lake Ohnuma in October 2010. Cyanobacterial colonies were washed twice in sterilized distilled water utilizing buoyancy of *M. aeruginosa*. Experimental water samples were collected from a reed belt of Lake Ohnuma and a water plant bed of Junsainuma-Lake. Collected waters were diluted and inoculated to washed cyanobacterial bloom waters with concentrations of 1/10, 1/100, and 1/1000 with supplements of 1/10 strength CT medium for algae. *M. aeruginosa* increased in bottles with no inoculation of experimental waters. In contrast, *M. aeruginosa* drastically decreased to 2~28% of the initial density during 3-week incubation when inoculated with experimental waters, and other diatoms, chlorophytes and harmless cyanobacteria increased. A new environment-friendly strategy for controlling *Microcystis* blooms is presented, i.e. large-scale developments of reed community and water plant zones in lakes with *Microcystis* blooms, and transmission of waters of these zone to *Microcystis* blooming areas.

Keywords: *Microcystis aeruginosa*, algicidal bacteria, reed belt, water plant, environment-friendly mitigation.

Introduction

Toxic cyanobacterial blooms have occurred with increasing frequency in lakes, ponds and drinking water reservoirs in almost every part of the world (Cronberg and Annadotter 2006). *Microcystis aeruginosa* is one of the most toxic bloom-forming species that causes deterioration of water quality to give negative impacts on animals and human beings, and decreasing aesthetic value of affected water. Therefore, the development of mitigation strategies to reduce and/or prevent occurrences of the cyanobacterial blooms in water systems is an urgent and serious need. As environment-friendly tools for control of toxic cyanobacterial blooms, algicidal bacteria have gathered attention to be promising agents against the blooms since they are abundant in aquatic ecosystems, proliferate rapidly, and are sometimes prey-specific (Daft et al. 1975; Kim et al., 2007; Imai and Yamaguchi 2012).

It was newly discovered that huge number of algicidal bacteria are attaching at the surface (in biofilm) of submerged reed stems in water and in adjacent surrounding water in coastal area of Lake Biwa, Japan (Imai 2010). And further, we newly isolated algicidal bacteria (strains of *Agrobacterium vitis*) active against *M. Aeruginosa* from the surface (in biofilm) of the water plant *Egeria densa* (Imai et al. 2012). In the present study, we newly discovered a phenomenon of controlling activities against *M. aeruginosa* by utilization of microorganisms in waters of a reed community (*Phragmites australis*) and a water plant bed in lakes in Hokkaido area of Japan. And we discuss about a potential importance of reed communities and water plant zones for the practical regulation of *M. aeruginosa* blooms.

Material and Methods

Samplings were made in Lake Ohnuma District on 28 October 2010. A cyanobacterial bloom sample mainly consisting of *Microcystis aeruginosa* in surface water was collected using 500-mL sterilized bottles at the port of Lake Ohnuma (OP in Fig.1). Cyanobacterial colonies in the top of bottle were

carefully collected with a sterilized pipette, put into the sterilized distilled water, and washed twice by leaving 15 minutes utilizing the buoyancy of *M. aeruginosa*. Experimental water samples were collected from a reed belt of Lake Ohnuma and a water plant zone of Junsainuma-Lake (OC and JL in Fig. 1) on the same date. Main species of water plants are *Trapa japonica*, *Brasenia schreberi*, *Myriophyllum spicatum*, *Utricularia vulgaris*, *Nymphaea* spp., etc. These waters were diluted in 10-fold series and inoculated to washed cyanobacterial bloom waters with the concentrations of 1/10, 1/100, and 1/1000 supplementing with 1/10 strength CT medium for eliminating nutrient depletion stress. Non-washed bloom samples were tested for comparison. Samples receiving no addition of water from the reed belt or water plant zone served as negative controls for the experiments. Cells of *M. aeruginosa* and other microalgae were enumerated once per week. Total algal biomass (*in vivo* fluorescence), total bacteria (epifluorescence microscopy after DAPI-staining), and algicidal bacteria (microplate MPN method: i.e., incubation of *M. aeruginosa* donated by Professor S. Tsujimura, Kyoto-Gakuen University) with the addition of diluted samples after 1μm filtration (Imai et al. 1998) were also determined.

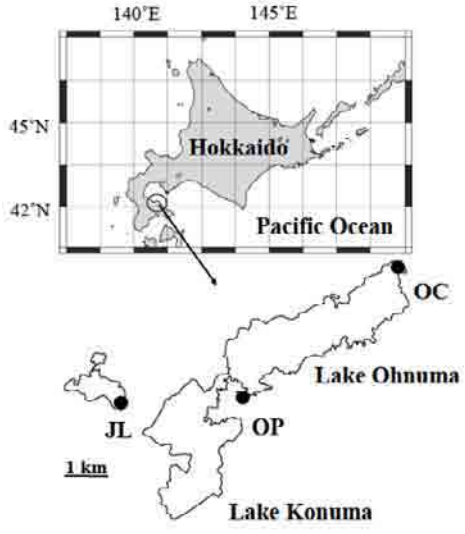

Fig. 1. Location of the sampling stations of Lake Ohnuma and Junsainuma-Pond in Hokkaido, Japan. OP: Ohnuma port, OC: Ohnuma camping site, JL; Junsainuma-Lake.

Results and discussion

Figure 2 shows the result of control (no addition of experimental waters from OC and JL) in the incubation experiments of *Microcystis aeruginosa* bloom water. In the bottle of washed bloom sample (W Control), *M. aeruginosa* cell density changed between $2.8x10^6$ and $5.7x10^6$ cells mL^{-1}, and between $1.1x10^6$ and $2.8x10^6$ cells m L^{-1} in the non-washed bloom sample (NW Control). The fluorescence of phytoplankton (total biomass) showed some increase during the experiment due to the consumption of nutrients initially added into the bottle (1/10 strength CT medium). Total bacteria fluctuated between $3.1x10^6$ and $1.8x10^7$ cells m L^{-1}. *Microcystis*-killing microorganisms were detected at 4.0-10.7 MPN m L^{-1} in the W Control and 0-16.9 MPN m L^{-1} in the NW Control.

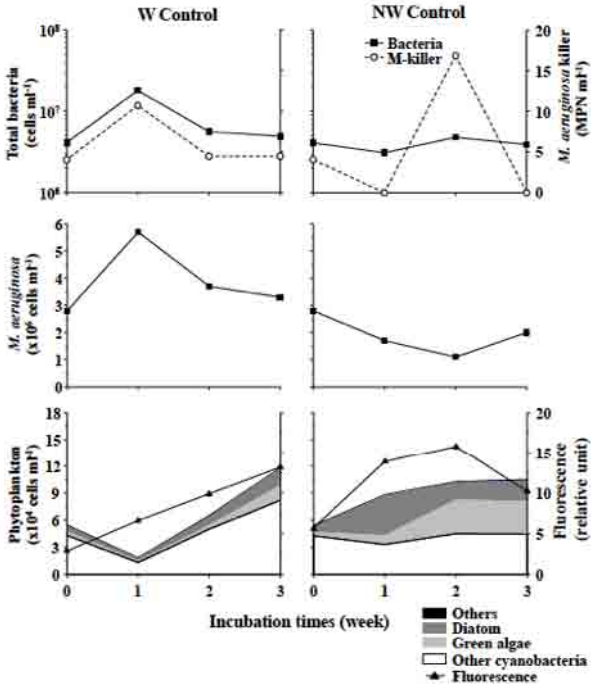

Fig. 2. Results of incubation of cyanobacterial colonies with no addition of experimental waters. "W" means washing treatment of cyanobacterial colonies with sterilized distilled water and "NW" non-washing of the colonies for the experiments. Note "M-Killer" indicates the Microcystis-killing bacteria detected with the microplate MPN method, and the "other cyanobacteria" denotes the number of algal cells excluding *M. aeruginosa*.

Figures 3 and 4 give the results of the mixed incubation experiment of the *M. aeruginosa* bloom sample and the experimental water collected at the sites of a reed belt OC and a water plant zone JL.

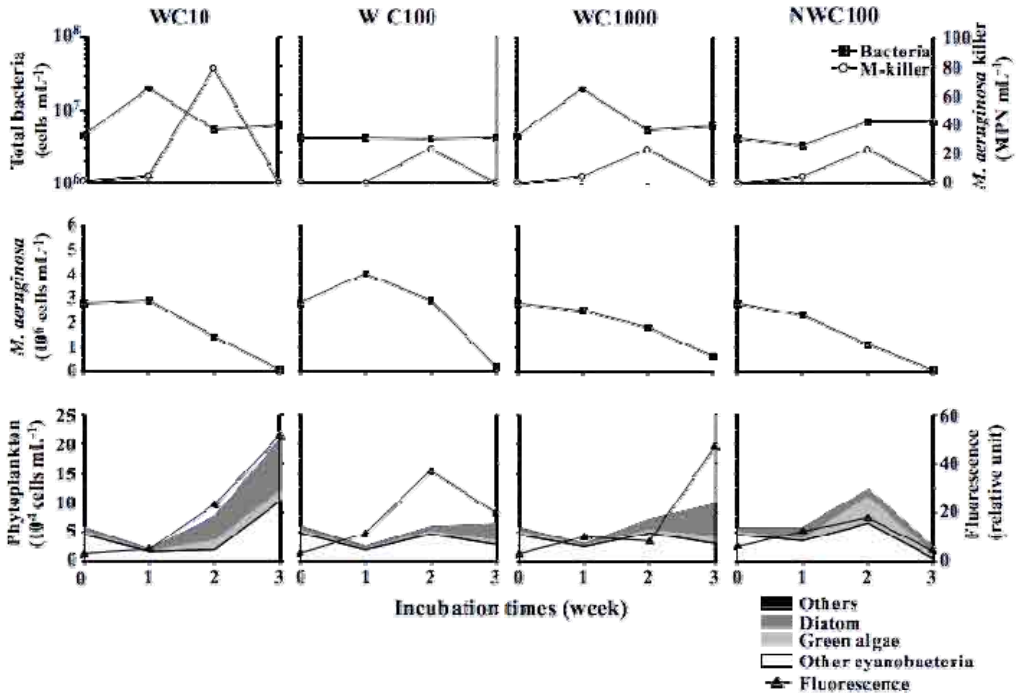

Fig. 3. Results of the mixed incubation experiment of the *M. aeruginosa* bloom sample and the experimental water collected at a reed belt of the site OC. Experimental water was diluted in 10-fold series and added to the bloom water at the concentration of 1/10 (WOC10), 1/100 (WOC100) and 1/1000 (WOC1000). WOC100 means the density of WOC100 and non-washing of the bloom material.

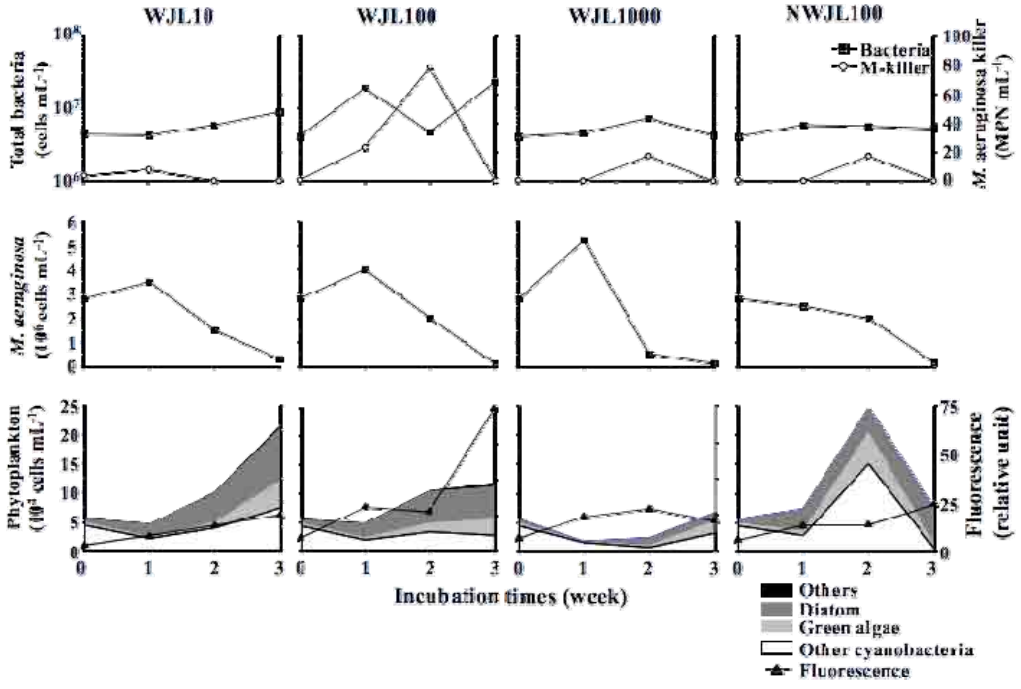

Fig. 4. Results of the mixed incubation experiment of the M. aeruginosa bloom sample and the experimental water collected at a water plant zone of the site JL. Experimental water was diluted in 10-fold series and added to the bloom water at the concentration of 1/10 (WJL10), 1/100 (WJL100) and 1/1000 (WJL1000). NWJL100 means the density of WJL100 and non-washing of the Bloom material.

In Fig. 3 of the results obtained in reed belt of the site OC, the total number of bacteria ranged from $4.1x10^6$ to $1.9x10^7$ cells mL^{-1}. Algicidal bacteria generally showed a peak at the second week and the maximum value was 78 MPN mL^{-1}. The cell densities of *M. aeruginosa* revealed a decreasing trend, and the percentages of decrease were 78.1-98.1% (Table 1). The total phytoplankton biomass increased 20 times or more as compared with the initial value. The increased phytoplankton taxa were diatoms (especially *Nitzschia*, *Fragilaria*), chlorophytes (*Ankistrodesmus*, *Scenedesmus*) and cyanobacteria (*Phormidium*, *Aphanocapsa*) excluding *M. Aeruginosa.*

Table 1. Decreased percentage of *Microcystis aeruginosa* cell density in the mixed incubation experiments (see Figs. 2 − 4). Percentage values were estimated by the difference between the final week data and the initial data.

Experiments	Percentage decrease
WOC10	97.8
WOC100	94.0
WOC1000	78.1
NWOC100	98.1
WJL10	89.0

In Fig. 4 of the experiment in water plant bed of the site JL, the total number of bacteria ranged from $4.1x10^6$ to $2.3x10^7$ cells mL^{-1}. Algicidal bacteria revealed a peak at the second week and the maximum value was 78 MPN mL^{-1} despite a possibility of underestimation due to omitting particle-associated algicidal bacteria (Inaba et al. 2014). The cell densities of *M. aeruginosa* decreased toward the 3rd week, and the decreased percentages were 89.0 − 96.2% (Table 1). The Phytoplankton biomass showed an increase of at least 20-fold as compared with the initial value, We speculate that high levels of moribund algal cells easily attacked by algicidal bacteria were present in non-washed material, causing the near total loss of phytoplankton biomass by the end of week 3 (Figs. 3 and 4). The increased phytoplankton taxa were diatoms (especially *Nitzschia*), chlorophytes (*Chlamydomonas*) and cyanobacteria other than *M. aeruginosa*.

It is possible that some unknown factors basides algicidal bacteria contributed to the decline of *M. aeruginosa*, since such bacteria were rather abundant in controls but did not completely remove the target cells, as were the cases in other experimental treatments.

The results of Figs. 3 and 4 appeared to show a similar pattern, i.e. the waters from the reed community and the water plant zone have an ability to reduce the cell density of *M. aeruginosa*. Therefore, the restoration and/or big scale creation of reed belts and water plant zones are considered to have a potential to prevent the occurrences of *M. aeruginosa* blooms in lake systems.

Eutrophication in Lake Ohnuma is progressing due to inflow of eutrophicated water from non-point source originated by agriculture and livestock industry (Yoshimura et al. 2000). *Microcystis aeruginosa* blooms started to occur from the 1980s along with the eutrophication (Takano et al. 1998). It is presumed that the reed communities and water plant zones supply algicidal bacteria (hopefully *Microcystis*-killer) and change the species composition to diatoms and chlorophytes, leading to prevention of harmful *Microcystis* blooms.

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Blooms of the colonial green algae *Botryococcus braunii* Kützing associated with massive fish mortality in Nozha Lake, Alexandria, Egypt

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Abstract

Lake Nozha is a natural fishpond where a controlled fertilizing programme, to increase its productivity as natural food for fishes, is applied. The lake is site of recurrent algal blooms, mainly of blue-green algae. The dramatic change in species composition in 2012 revealed the predominance of the non-toxic green algae *B. braunii,* which formed intermittent blooms. Irregular sampling was operated for six months (January to June 2012) at five selected stations. The green-yellow water, turned to brown, covered the entire lake during the calm periods in January, when *B. braunii* (up to 55.3×10^3 colonies L^{-1}) was the sole component of the phytoplankton community and caused mass mortalities of mullets, tilapias, bayads, carps and catfish (estimated losses, 3000 US \$ d^{-1}). This is the first report of harmful blooms of *B. braunii* in Nozha Lake and the environmental conditions associated with it. The fish-killing mechanism was not identified.

Keywords: *Botryococcus braunii*, fish mortality, harmful algal blooms, Lake Nozha (Alexandria)

Introduction

Nozha Lake, Alexandria, is an enclosed, nearly circular freshwater body of 5.5 km^2 and average depth of 2.1 m. The Nile river, via the Mahmoudia Canal, empties into the lake through the Feeding Canal. In 1939, Nozha Lake was isolated by an embankment from Lake Mariut and since 1960 is exploited as a fishpond, with a production of 200-250 $t \cdot y^{-1}$ of fish. In the last two decades, the lake has suffered intensive pollution from untreated sewage and industrial and agricultural discharges. A controlled fertilizing programme to increase productivity as a natural fish food led to increased nutrient levels and created favorable conditions for recurrent algal blooms. Cyanobacteria (*Anabeana*, *Microcystis* spp.) bloomed in 1986, 1994, and 2003, associated with massive fish mortality (Lake Authority, pers.com.). *Botryococcus braunii* Kützing (Chlorophyceae) is a cosmopolitan green unicellular microalgae widely distributed in temperate, tropical and continental waters (Qin 2005). Mass production of *B. braunii*, which forms aggregates of up to 0.5 mm in diameter, has been proposedas as a renewable source of biodiesel (Banerjee *et al*. 2002; Chiang *et al*. 2004; Ikawa 2004; Qin 2005), a reducer of CO_2 emissions (Sawayama *et al*. 1994) and an eco-friendly way to produce lipids

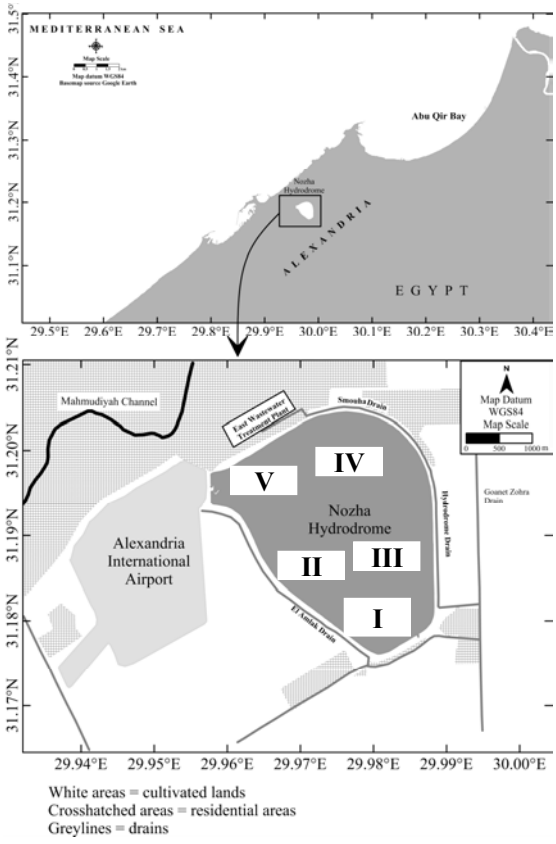

Fig. 1. Sampling stations in Nozha Lake (from Rifaat and Ahdy 2011)

and other bioactive compounds. Great variability in hydrocarbon yields among different species and strains of Botryococcus is attributed to growth conditions (Lee *et al*. 2010; Samori *et al*. 2010). This is the first study to determine environmental conditions associated with the development and maintenance of harmful blooms of *B. braunii* in Egyptian lakes.

Material and Methods

The study was part of a monitoring program operated at five stations (Fig. 1) during January-June 2012. Sampling was restricted to the water discoloration periods. Measurements were taken of surface water temperature (thermometer with accuracy + 0.1°C), salinity (calibrated refractometer S/Mill), and dissolved oxygen (Winkler method, APHA 1975). Surface water samples for nutrients determination were immediately filtered (Whatman GF/F) and kept frozen until analyses. Inorganic nitrate, phosphate and silicate concentrations were determined according to Strickland and Parsons (1972). Cells density (colonies L^{-1}) was determined from three 1-mL replicates per sample and counted (x100) with a counting chamber.

Results and Discussion

Monthly averages of water temperature (T) ranged from 13°C in January to 28°C during abnormal hot weather in June; salinity (S) between 1.4 and 2.5 psu, and pH between 7.8 and 8.9, but values of 8.7 and 9.5 were recorded during bloom maxima in January and February. Dissolved oxygen varied from 7.2 to 9 mg l^{-1}, but mass occurrence of *B. braunii* in January-February raised it to extreme values (10.6-11.6 mg l^{-1}), affecting fish survival and biodiversity. The high alkalinity changes (300-420 mg l^{-1}) observed may explain the onset of quasi mono-specific blooms, damaging phytoplankton diversity. Studies in Australia revealed optimum T and S for growth were 23°C and 8.8 psu, but a wide tolerance to T, up to 33°C, have been recorded elsewhere (Qin 2005). In other studies, optimal conditions for *B. braunii* growth were 20-25°C (Casadevall *et al*. 1985), and 17-85 mM salinity (Ranga Rao *et al*. 2007). The latter wrote that salinity is one of the factors influencing increased lipid content. *B. braunii* was also found to adapt to a wide pH range (Dayananda *et al.* 2005). Our data supports Komárek and Marvan (1992) who wrote that *B. braunii* grows under a wide spectrum of conditions. The major bloom peaks in January ($55.3x10^3$ colonies L^{-1}) and February ($30.2x10^3$ colonies L^{-1}) support the conclusion that the *B. braunii* strain from Alexandria is adapted to low temperature. Yet, there were other minor peaks ($20.5x10^3$-$25.5x10^3$ colonies L^{-1} between site I and III) in May at 23°C, similar to those reported for the China strain 1 (Qin 2005).

Nutrients: The bloom peaks in January-February consumed most of the available nitrate, reducing it to 0.03 and 0.05 mg l^{-1}, respectively, while the minor peak in May occurred with 0.9 mg l^{-1}. The same observation was found for phosphate (0.04 and 0.05 mg l^{-1}, respectively), and 0.1 mg l^{-1} in May. Wide fluctuations (0.06-0.9 mg l^{-1}) of silicate suggest it was a non-limiting factor. High concentrations of nutrients and alkalinity were likewise observed during a *B. braunii* bloom in Banglang reservoir, Thailand (Ariyadej *et al.* 2004). Yet, deficiency of nitrogen favors lipid accumulation (Ben-Amotz and Tornabene 1985), and, experimentally, the maximum lipid content of *B. braunii* was 63% with 0.04 mM nitrate (Choi *et al*. 2011). According to Banerjee *et al*. (2002), the alga was able to consume PO_4^{3-} present at quite low levels (0.02 $g \cdot m^{-3}$), and it requires very low levels of nitrate and phosphate to produce the highest hydrocarbon content (Marsh 2008). The minimum concentrations of nitrate and phosphate with the bloom peaks may indicate its ability for self-purification processes of natural waters, similar to other microalgae (Craggs *et al*., 1997). It is believed that lipid production with such blooms varies not only among strains but also with environmental conditions, e.g. temperature (Lupi *et al*., 1991), photoperiod and light intensity (Brenckman *et al*., 1989), salinity (Vázquez-Duhalt *et al*. 1991; Derenne *et al*. 1992) and nitrogen levels (Sawayama *et al*. 1992; Singh and Kumar 1992). The effect of these factors on growth and lipid content of *B. braunii* under various experimental conditions was proved (e.g., Qin 2005).

Changes in *B. braunii* population*:* Rust-coloured aggregates were frequently observed on the lake surface. Microscopic examination confirmed these to be of colonial green alga, *Botryococcus braunii,* which formed dense intermittent blooms. Nozha Lake water is characterized by its green-yellow to brown color, changing to orange/rust with blooms and associated floating mats. The rust color, directly related to bloom density (Chiang *et al*. 2004; Ikawa 2004) is a common feature (Qin 2005). The

Fig. 2. Golden-yellow colonies of *B. Braunii* (top) associated with fish mortality (bottom).

golden-yellow color is due to extracellular oil on the colonies. The visible blooms covered the entire lake surface during calm periods, and caused massive fish death in January (Fig. 2) particularly of *Tilapia* sp. (estimated losses of 3000 US $ d^{-1}). Spatial variability in distribution of *B. braunii* (Fig. 3) showed that maximum values were always found in site I (mean density $23.75x10^3$ colonies L^{-1}) and density gradually decreased towards the middle part of the Lake but sharply towards the west ($11.55x10^3$ colonies L^{-1}, Site V). Highest numbers of *B. braunii* were found in January (mean density $34.72x10^3$ colonies L^{-1}) and February ($22 x10^3$ colonies L^{-1}), with lower values in May ($19.42 x10^3$ colonies L^{-1}), and the lowest in March and June ($10.4x10^3$ and $7.3x10^3$ colonies L^{-1}).

Co-occurring phytoplankton species during the blooms included a few cells of green algae (*Chlorella* sp.**,** *Pediastrum simplex, Scenedesmus quadricauda*), Cyanobacteria (*Anabaena spiroides, Anabaena* sp., *Chroococcus* sp., *Microcystis* sp., and *Oscillatoria* sp.) and pennate diatoms (*Nitzschia* sp.). This suggests that *B. braunii* may exert allelopathic effects on other phytoplankters (Chiang *et al.* 2004). High pH in Nozha Lake may help to explain the quasi mono-specific nature of the blooms; this also enhances the formation of free fatty acids, that are known to be more toxic to aquatic organisms with high pH values (Proctor 1957).

Increased urbanization and socio-economic activities around Nozha Lake are expected to increase the risk of harmful blooms unless urgent improvements in the physicochemical properties (especially pH) of the lake are attained. Improved knowledge on conditions leading to *braunii* dominance willl be helpful for its mass production as an optimum hydrocarbon source in Egypt.

Unfortunately we were not able to determine the mechanism causing fish mortality.

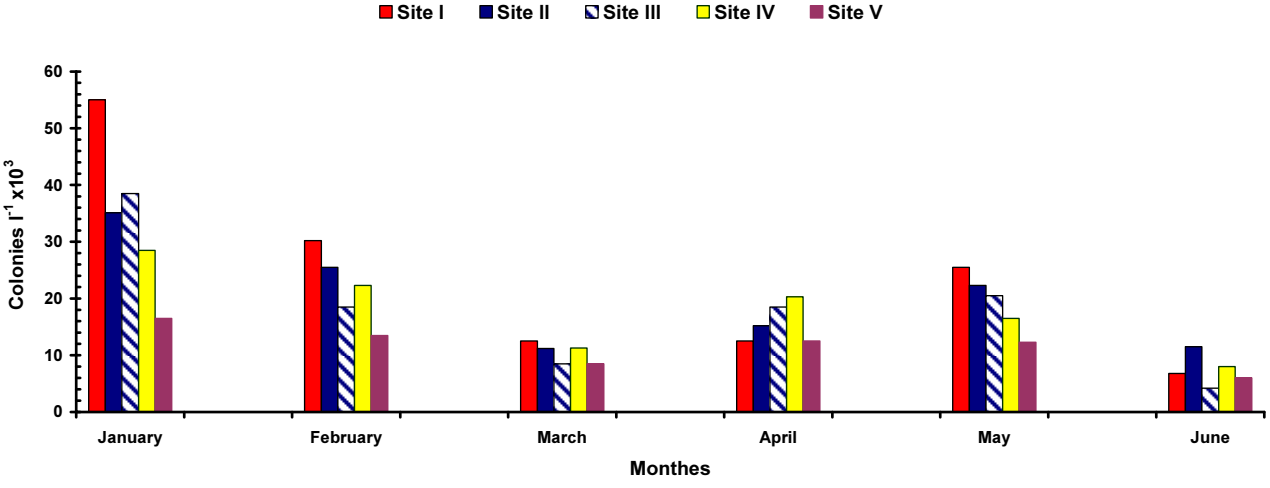

Fig. 3. Monthly variations of *B. braunii* (January-June, 2012)

Considering that *B. braunii* is not a toxin producer, death may have been caused by abrupt changes in some physico-chemical parameter (dissolved oxygen, viscosity) or by the production of some reactive oxygen species. This is the first report about *B. braunii* blooms in Nozha Lake, Alexandria.

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Occurrence of red coloured *Planktohrix* (Cyanophyta) species in Estonian lakes

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Abstract

During a national environmental monitoring programme between 2006 and 2011, samples for phytoplankton analyses were collected in 96 lakes three times per year, between May and August. A total of 634 samples were collected where phytoplankton species were identified at genus or species level. Red type *Planktothrix* was observed only in one eutrophic lake, Nõuni, in 2008 where in May, two weeks after ice-break, an extremely high surface (epilimnion) biomass (37.4 mg L^{-1}) was estimated. In July, the biomass peak had moved to the metalimnion and reached a maximum of 41.4 mg L^{-1}. In August, similar values were measured in epi- and metalimnion (9.9 and 8.3 mg L^{-1}, respectively). Outside of monitoring program red coloured *Planktothrix* has been observed at least two small eutrophic stratified lakes in autumn 2008. In lake Agali filaments of *Planktothrix prolifica* were in the metalimnion together with filamaents of *Planktothrix agardhii* and in lake Kuningvere an intensive surface scum occurred caused also by *Planktothrix prolifica*. The detection of red type *Planktothrix* in lake Nõuni and Kuningvere was possible by naked eye due to discoloration of surface waters. In lake Agali unpreserved sample were examined and therefore the colouration of filaments were visible. This let us presume that *P. prolifica* and also *P. rubescens* probably have a broader distribution that has simply not been documented. Overlooking is common due to preservation of samples with Lugol's solution, which changes the filaments coloration and renders differentiation between green and red coloured types impossible. This is most likely in samples taken from deepest layers where water discoloration are not recognizable. The aim of this paper was to give an overview of the presence of red type *Planktothrix* species in Estonian lakes.

Keywords: Lake Nõuni, *Planktothrix prolifica, Planktothrix rubescens*, cyanobacteria

Introduction

Estonia, located in Northeast Europe, borders the Baltic Sea on the west, the Gulf of Finland on the north, Latvia on the south, and Russia on the east. It has a surface of 45,226 km^2 and about 2,308 small lakes with an aquatory > 1 ha representing 4.8 % of its territory (Tamre 2006). According to the limnological typology elaborated by Mäemets (1974), eight lake types can be found in Estonia: oligotrophic (8% of the lakes), semidystrophic (6%), dystrophic (6%), eutrophic (36-37%), mixotrophic (36-37%), siderotrophic (0.2%), halotrophic (1.4%) and alkalitrophic (2.6%).

Cyanobacterial blooms have been a regular phenomenon in Estonian lakes (Olli 1996). Moreover, these blooms are reported to have increased in frequency, biomass, and duration in recent decades, primarily in response to anthropogenic eutrophication (Ott and Kõiv 1999). Biomasses over 8 mg L^{-1} made up by 20 different cyanobacterial species have been detected in approximately 15% of its lakes (Rakko *et al*. 2008).

The red type *Planktothrix* can be classified separately from the green type, and coloration of cells can be therefore used for taxonomic identification (Komárek and Komárková 2004) provided Lugol's solution is not used to preserve the samples. *P. prolifica* and *P. rubescens* differ from each other mainly by their dimensions but a wide variability of this character has been shown (Davis *et al*. 2003b). Up to now it seems that both species have slightly different geographical distribution, with overlappings in some areas. *P. rubescens* occurs in colder lakes from central and northern Europe and North America, whereas *P. prolifica* occurs in central, northern and northeastern Europe and northeastern USA (Komárek and Komárková 2004).

The aim of this paper was to give an overview of the presence of red type *Planktothrix* species in

Estonian lakes. In addition, a detailed description is given of the seasonal development of *P. prolifica* blooms, starting under the ice in winter.

Material and methods

The subject of this study were the lakes located in the mainland but not those in the islands. Sampling was adapted to the lake type (oligotrophic, semidystrophic, dystrophic, eutrophic, mixotrophic). "Red type" *Planktothrix* were observed in three hard water eutrophic lakes. Lake Nõuni (surface 82.1 ha, mean depth 6.1 m, maximum depth 14.7 m, drainage area 6.3 km^2), Lake Agali (surface 12.8 ha, mean depth 5.8 m, maximum depth 19.5 m, drainage area 1.1 km^2) and Lake Kuningvere (surface 24.9 ha, mean depth 4.8 m, maximum depth 7.4 m, drainage area 3.1 km^2).
Samples for phytoplankton analysis in Lake Nõuni were collected at the deepest point of the lake with a van Dorn sampler. Phytoplankton samples were immediately preserved with Lugol´s solution and kept in the dark at 4 ºC until counting. Algae cells were counted using an inverted microscope (Hund Wetzlar) according to the Utermöhl (1958) method at a magnification of 100-400X. Filament biovolumes were calculated using geometrical models according to Hillebrand *et al*. (1999). In the case of filamentous cyanobacteria, the length of at least 50 trichomes per sample was measured and the mean length was used for biomass calculations. Samples in Lake Agali were collected at the deepest point of the lake with a van Dorn sampler and kept unpreserved in cool and dark. Sample from Lake Kuningvere were taken by hand from shore and filaments kept unpreserved in cool and dark. For both samples determination took place at the same day using an inverted light microscope (Nikon Eclipse TS100).

Results and Discussion

The cyanobacterial genus *Planktothrix* belongs to the planktic filamentous phormidiacean type. Among other cyanobacteria, species of *Planktothrix* have been frequently responsible for water blooms in eutrophic lakes (Nõges and Ott 2003; Rakko 2009). In Estonia, green type *Planktothrix agardhii* was common in both, shallow and stratified eutrophic lakes (Rakko *et al*. 2008). Other "green type" species (*P. isothrix*, *P. clathrata*) were not prevalent and appeared in low to moderate numbers in the hypolimnion in stratified lakes or rarely in the water column in shallow lakes.
During the monitoring programme period, 2006-2011, the presence of "red type" *Planktothrix* was recorded only in one lake (Lake Nõuni) in 2008. Visible rust-coloured water consisted of high numbers of filaments of the "red type" *Planktothrix prolifica* (Fig. 1). Water transparency ranged from 0.7 to 2.5 m from May to September. The bloom development pattern was the opposite of typical cyanobacterial blooms in Estonian temperate lakes (Rakko *et al*. 2008). In the middle of May, two weeks after ice break, the highest estimated biomass value (37.4 mg L^{-1}) was found in the epilimnion (Table 1). In July a slightly increased biomass peak (41.4 mg L^{-1}) had moved to the metalimnion. In August, both the epi- and metalimnion biomass (7.1 and 6.6 mg L^{-1} respectively) showed a considerable decline. A considerable biomass was observed in the hypolimnion in May, but not in July and August. Intensive spring blooms could only be supported by development of filamentous populations under the ice before its melting. Additionally, deep autumn

Fig. 1. Mass occurrence of *P. prolifica* in the stratified eutrophic Lake Nõuni (top) and Secchi disc reading (bottom) two weeks after ice-melting on 14 of May 2008.

mixing, which brings nutrients to the epilimnion and a change of light conditions are the factors favouring growth and biomass increase of phycoerythrin containing *Planktothrix* species during winter (Davis *et al*. 2003a).

Outside of the monitoring programme *Planktothrix prolifica* was observed at two eutrophic stratified lakes. In Lake Agali most of red coluored *P. rubescens* filaments layed on the metalimnion with filaments of green coluored *P. agardhii*. In Lake Kuningvere surface scum occurred and no green coloured *Planktothrix* type were not identified.

Planktothrix rubescens has been reported several times in Lake Nõuni and at least once in the stratified hypertrophic Lake Juusa (Laugaste 2006). In both lakes ice colouration was observed in late winter or early spring. Unfortunately species were identified by their colouration not by dimensions and therefore those data are not reliable. However, mass occurrences have not been noticed during the vegetative period. *Planktothrix prolifica* had not been documented before 2008 and this is the first report of that cyanobacterium in Estonian lakes. In Europe, this species has been found in northeastern Poland, northern Germany, the Netherlands, Czech Republic and central Spain (see Kaštovský *et al*. 2010 and references therein). So far, there are no reports of this species in the Baltic countries.

P. rubescens and *P. prolifica* probably have a broader distribution that has simply not been documented. Occurrence of red-coloured species during this study just in one lake might be inaccurate. Typically "red type" species are located in the metalimnion, a transition area between the warm nutrient-poor epilimnion and the colder nutrient-rich hypolimnion (Davis *et al*. 2003a; Micheletti *et al*. 1998). For Lake Nõuni, visible red water colouration indicated the presence of red-coloured *Planktothrix*. On the other hand, metalimnion populations are not visible by naked eye and in samples preserved with Lugol's solution, discrimination between green-coloured and red-coloured types is impossible. Therefore *P. rubescens* and *P. prolifica* probably have a broader distribution that has simply not been documented. The reason why both species have been overlooked is that preservation of samples with Lugol's solution causes cells bleaching and makes it impossible to distinguish red- and green types. Only filament size and shape are not sufficient for identification. Samples could be preserved with 2% of formalin which does not discolour the cells. Differently from Lugol's solution, formalin does not make the cells heavier and then they do not settle easily to the bottom of the counting chamber. Therefore for cell counting with Utermöhl's technique, samples must be preserved in Lugol solution.

Intrageneric diversity of *Planktothrix* species, which is important for identification, is complicated and the classification at the species level is difficult. Since a wide variation in filament length has been shown for both red type species, the differentiation may be problematic in some cases. According to Komárek and Komárková (2004) cell width of *P prolifica* ranges from 2 to 5.8 µm and for *P. rubescens* from 3.9 to 9.4 µm. During a study (2008-2009) with living samples, in one monitored and two non-monitored lakes, the range of filament width was 4-4.3 µm, 4.6-5.3 µm and 3.6-4.0 µm for Lake Agali, Lake Kuningvere and Lake Nõuni respectively. Since the average filament width was always narrower than 6 µm and tapered, morphology of the morphotypes observed matched better with the description of *P. prolifica* than with that of *P. rubescens*. However, morphological characters can

Table 1. Parameters measured in Lake Nõuni in 2008. (Epi - epilimnion, Meta - metalimnion, Hypo - hypolimnion).

	Transparensy	Strata	Depth	Chla	Biomass	Biomass of *P. prolifica*	TotP	TotN	Oxygen
	m	-	m	µg/l	mg/l	mg/l	µg/l	µg/l	mg/l
14 May	0.9	Epi	0.5	22	40.8	37.4	43	870	12.56
		Hypo	11	9	7.4	7.1	3	660	7.17
11 July	0.7	Epi	0.5	6.8	10.1	8.9	2	640	9.9
		Meta	5	51	41.7	41.3	64	790	0.9
		Hypo	11	2.2	0.2	0.04	240	1500	0
15 August	1.9	Epi	0.5	7.1	9.9	7.1	na	Na	9.4
		Meta	5	6.3	8.3	6.6	na	Na	7.3
		Hypo	11	4.1	0.3	0.01	na	Na	0

vary under different environmental conditions. Therefore, for reliable identification frequent samples are needed to measure morphological features, both in young and old populations. 16S rRNA sequencing can also be used, but that was not the purpose for a routine monitoring.

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First of all we would like to thank ISSHA International Scientific Committee and Korea Local Organizing Committee for a very well organized conference. The material for this article was collected and analyzed as part of the Estonian state monitoring subprogram "Hydrobiological monitoring of Estonian small lakes". Authors would like to thank all colleagues who took part in this investigation.

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Impacts of HABs, management, and mitigation of HABs

Extended blooms of *Karenia concordia* and other harmful algae from 2009 to 2011 in Wellington Harbour, New Zealand

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Abstract

The first *Karenia concordia* bloom in Wellington Harbour occurred in early April 2009, with a relatively small build-up of *Vicicitus globosus* cells observed in May in the same year. A year later in May 2010, *K. concordia* was found to be part of a larger bloom which also included *Heterosigma akashiwo*, *V. globosus* and *Pseudochattonella* cf. *verruculosa*. This multispecies bloom dominated by *H. akashiwo* apparently contributed to a mass mortality of cage-reared fish at NIWA's Aquaculture Centre, in Mahanga Bay, Wellington. More *K. concordia* and *V. globosus* blooms were recorded in May/June 2011. However, no further bloom was detected in 2012. Physical and chemical conditions contributing to extended blooms from 2009 to 2011 in Wellington Harbour are discussed.

Keywords: *Karenia concordia*, fish kills, harmful algal blooms. New Zealand

Introduction

Over the past 30 years numerous algal blooms recorded in Wellington Harbour (Fig. 1) were largely dominated by harmless diatom, dinoflagellate and flagellate species (Chang unpubl. results). Several toxic and potentially harmful species, e.g., *Karenia brevisulcata*, *Chattonella marina*, *V. globosus*, *P.* cf. *verruculosa*, *H. akashiwo*, *Gymnodinium catenatum*, *Alexandrium minutum*, *Dinophysis acuminata* and *D. acuta* have also been recorded in the harbour. Even though some had built up to bloom proportions, none had ever been associated with toxic outbreaks (Chang *et al*. 2012; Chang unpubl. results). The *K. brevisulcata* bloom reported in the summer 1998, however, was the first to contribute to mass mortalities of marine fauna, flora, and human respiratory syndrome in the harbour (Chang 1999; Chang *et al*. 2001). Eleven years later, in late summer 2009, another two toxic algal species, *K. concordia* and *V. globosus*, formed extended blooms, seemingly triggered by a massive discharge into the harbour of treated effluent caused by a ruptured sewerage pipe from March to May 2009. The former species was previously reported to cause mass mortalities of fish and marine fauna in Hauraki Gulf, on the north eastern coast of New Zealand (Fig. 1A) (Chang and Ryan 2004, Chang *et al*. 2008).

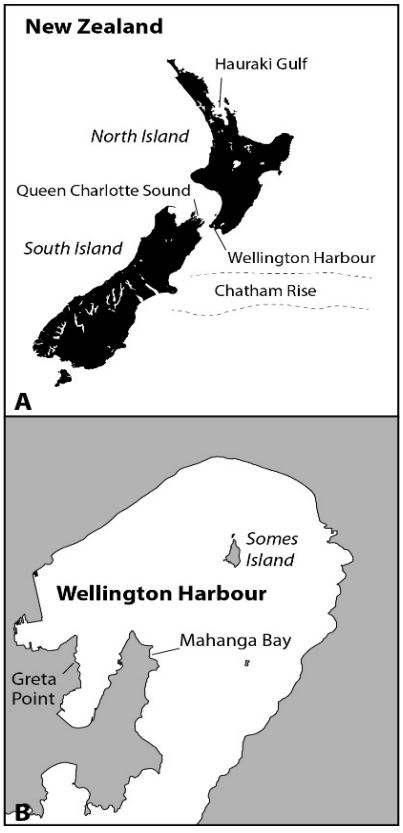

Fig. 1. Maps of (A) New Zealand, and (B) Wellington Harbour, showing the location of Mahanga Bay and where mortality of cage-reared fish was reported in 2010.

This study explored the extended bloom events of *K. concordia* and *V. globosus* from 2009 to 2011 which ended abruptly in 2012. A multi-species bloom including *H. akashiwo* and *P.* cf. *verruculos*a (Chang unpubl. results), related to a fish kill event in sea cages in May 2010 in Wellington Harbour, is also included in this study.

Material and Methods

The mean monthly temperature of daily maximum/ minimum and mean monthly daily wind run, in km, for four consecutive years from 2009 to 2012, were processed from data obtained at Wellington Airport (NIWA's National Climate Database). Monthly and three monthly mean of Southern Oscillation Index (SOI) were also prepared over the period from 2009 to 2012.

Discrete water samples from the surface were collected in 150 ml plastic bottles at weekly intervals from Mahanga Bay and at several sites in the harbour. But only cell concentrations of the few key harmful algal bloom (HAB) species recorded at Mahanga Bay are presented here.

All water samples collected were preserved in 1% Lugol's iodine solution. Identification and enumeration of phytoplankton species were carried out using a Nikon inverted light microscope, after settling 10 ml subsamples in sedimentation chambers for 24 h (Utermöhl 1958).

Results

Physical conditions

Temperature records obtained from Wellington Airport between 2009 and 2012, showed May and June of 2009 were the coldest months, while March and April of 2010 and May of 2011 were the warmest (Fig. 1). The Southern Oscillation Index (SOI) in 2009 was negative (*El Niño* weather conditions) while those of 2010 and 2011 were positive (*La Niña*) (Fig. 2). The condition in 2012 was neutral; temperatures from April to June were between the two 'extremes'. Although persistent strong southerly and south-westerly winds (gale-force at times), prevailed throughout 2009 (Fig. 3), there were two periods of relatively calm conditions, from February to early April and from July to August. Also relatively calm conditions were recorded between May and June of 2010 and between March and April of 2011. It was, however, very windy from June 2011 to July 2012.

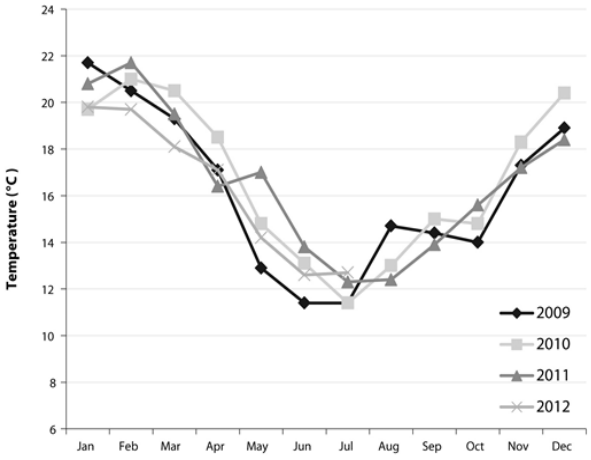

Fig. 2. Monthly mean temperatures from 2009-2012.

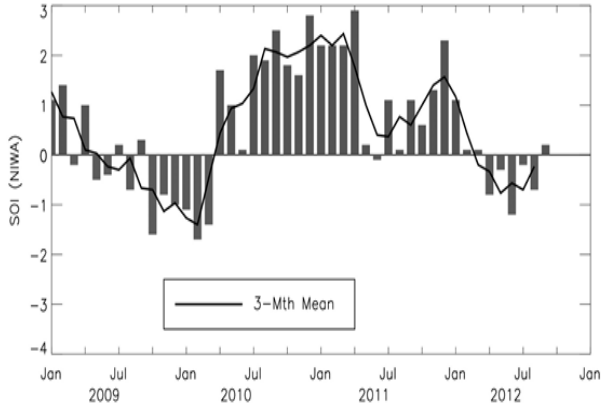

Fig. 3. Three monthly mean of Southern Oscillation Index (SOI) from 2009 to 2012.

Field observations

In March 2009, a small number of *K. concordia* (<100 cells l^{-1}) was detected for the first time in the harbour. This soon led to a major bloom in early April ($4.2x10^5$ cells l^{-1}) (Fig. 4), coinciding with the spillage of treated sewerage effluent (between March and May 2009). A bloom of a second species, *V. globosus*, in much lower concentrations, occurred in May 2009 ($1.4x10^3$ cells l^{-1}). Simultaneously other harmless species, e.g., *Eutreptiella* sp., *Noctiluca scintillans*, *Lauderia annulata*, *Chaetoceros* spp., also formed part of the persistent, widespread blooms in the harbour.

In May 2010, a multispecies bloom, including *H. akashiwo* ($4x10^6$ cells l^{-1}), *K. concordia* ($2.7x10^4$ cells l^{-1}), *V. globosus* ($9.1x10^3$ cells l^{-1}) and *P.* cf. *verruculosa* ($5x10^3$ cells l^{-1}) (Fig. 4) coincided with a mass mortality of cage-reared fish *(Polyprion*

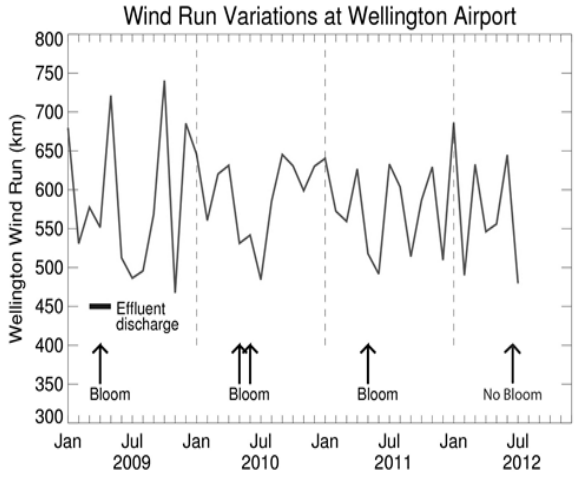

Fig. 4. Monthly mean of daily wind run (km) from 2009 to 2012.

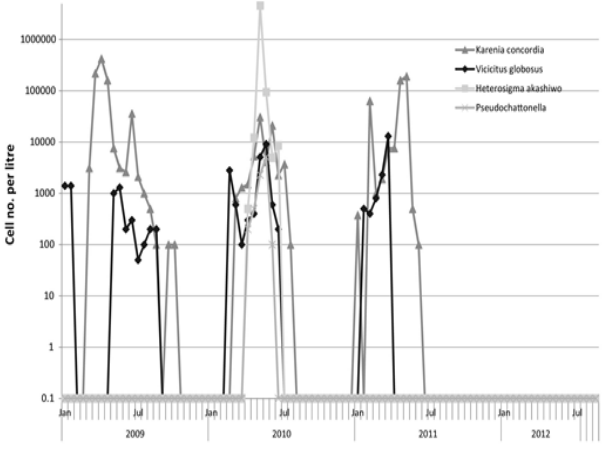

Fig. 5. Surface cell concentrations of *K. concordia*, *V. globosus*, *H. akashiwo* and *P.* cf. *verruculosa* recorded from 2009 to 2012 in the harbour.

oxygeneios) at NIWA's Aquaculture Centre in Mahanga Bay, Wellington Harbour.

In 2011, a *K. concordia* bloom resumed in February, which intensified in late April ($1.9x10^5$ cells l^{-1}), while *V. globosus* peaked in late March ($1.3x10^4$ cells l^{-1}), with the highest cell concentration recorded in three years. Cells of neither *K. concordia* nor *V. globosus*, however, were recorded from July 2011 to July 2012.

Discussion

Although persistent, strong winds appeared to characterise the 2009 *El Niño* weather conditions, a relatively calm period was noted between February and April 2009. The combination of relatively calm conditions and the nutrient enrichment resulting from the spillage of treated effluent from a ruptured sewerage pipe is thought to have contributed to a very intense *K. concordia* bloom in early April 2009.

From 2010 to 2011, blooms of both *K. concordia* and *V. globosus* also occurred in the calm periods, consistent with observations of the 1998 *K. brevisulcata* blooms in Wellington Harbour (Chang *et al*. 2001). However, *V. globosus* blooms recorded in the relatively warm and calm conditions of 2010/ 2011, were more intense than those of 2009, suggesting that bloom occurrence might be less dependent on the nutrient enrichment caused by the spilled, treated effluent than on physical, warm and calm conditions.

Prior to the May 2010 multispecies bloom, the harbour experienced a short period of persistent, warm and calm conditions. Coupled with surface nutrient enrichment caused by mixing during stormy weather in early 2010, *H. akashiwo*, apparently, was able to dominate the bloom in May. *La Niña* weather conditions are associated with warm, calm conditions (Basher and Thompson 1996). Similar observations were made by Chang *et al*. (1993) in relation to the 1989 *H. akashiwo* blooms in the Big Glory Bay, New Zealand. The June 2010, fish-killing, *P. verruculosa* bloom observed by MacKenzie *et al*. (2010) in the nearby Queen Charlotte Sound across Cook Strait, was also associated with similar persistent warm, calm, nutrient-rich conditions. Indirect evidence of the important role of weather conditions and surface mixing is found in 2012. The very windy conditions between June 2011 and July 2012 in the harbour appeared to end the possibility of any further blooms.

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Heterosigma akashiwo in the Salish Sea: Defining growth and toxicity leading to fish kills

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Abstract

The raphidophycean flagellate, *Heterosigma akashiwo*, (Y. Hada) Y. Hada ex Y. Hara et M. Chihara is responsible for extensive mortality of cultivated (pen-raised) finfish in Puget Sound, Washington, ($2-6 million in losses per episode), and may also affect wild nekton populations in the Salish Sea waters of British Columbia and Washington. Over the past two decades, these ichthyotoxic blooms have increased in both scope and magnitude, but neither the factors responsible for bloom initiation and maintenance, nor toxicity are clearly understood. The effects of cellular growth phase and the degree of nutrient sufficiency on the relative toxicity of these cultured cells were examined in a laboratory study with three different Puget Sound isolates of *H. akashiwo*. Toxicity, measured as decreased viability of a rainbow trout cell line (RTgill-W1) exposed to aqueous *H. akashiwo* extracts, was consistently elevated during the stationary phase of cell growth following the depletion of external N reserves due to phytoplankton uptake, whereas all three isolates were non toxic during their nutrient-replete, exponential phase of growth. These results are the first to demonstrate variability in toxicity as a result of physiological changes associated with growth stage or nutrient availability for cultures of *H. akashiwo.*

Keywords: *Heterosigma akashiwo*, Puget Sound, harmful algal bloom, gill cell assay

Introduction

The photosynthetic flagellate *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara & M. Chihara, 1987 is a marine raphidophyte that forms ichthyotoxic blooms. Blooms of *H. akashiwo* have been responsible for economically devastating kills of caged fish in aquaculture operations worldwide, including salmon farmed in the U.S., Canada, New Zealand, Chile, and Europe, and yellowtail and sea bream in Japan's Seto Inland Sea (Honjo 1993; Horner *et al.* 1997; Smayda 1998). There is also evidence for wild fish kills due to *H. akashiwo* (Hershberger *et al.* 1997; Horner *et al.* 1997), but the mechanism(s) of *H. akashiwo* toxicity to fish are not well understood. Several mechanisms of toxicity have been proposed ranging from extracellular hydrogen peroxide production, excessive mucus secretion, and the production of intracellular brevetoxin-like compounds. However there remains doubt about the efficacy of each: there is insufficient hydrogen peroxide produced to negatively impact species (Twiner and Trick 2000; Twiner *et al.* 2001); while fish appear asphyxiated most reports of fish death are rapid and mucus accumulation is generally the result of changes after death (Yang *et al.* 1995), and reports on brevetoxin-like components are based on HPLC co-chromatography and not sodium channel inhibition (Khan *et al.* 1997). In fact, isolated organics from *Heterosigma*, while having a molecular weight similar to brevetoxin, do not alter the sodium balance of isolated cell lines as does brevetoxin, but dramatically alters calcium homeostasis, indicating the substance as a unique bioactive metabolite (Twiner *et al.* 2005).

In the Salish Sea — the estuarine system on the North American west coast consisting of the Strait of Juan de Fuca, Puget Sound, and the Strait of Georgia — small-scale, ephemeral blooms of *H.*

akashiwo occur every year during the spring and summer, but other, less frequent, but massive blooms can cover hundreds of km^2 (Rensel *et al.* 2010). These blooms have together caused extensive damage ($2-6 million per episode) to the wild and net-penned fish of Puget Sound, Washington, and are believed to have increased in scope and magnitude over the past two decades. Although considerable progress has been made to elucidate the environmental factors that promote and contribute to the competitive success of this species, the primary fish-killing agent or mechanisms are still not identified, and there is no accepted and uniform chemical measure of the toxin content for this raphidophyte. Here we report the results of batch experiments designed to investigate the relationship between toxicity and cellular growth phase in three, Puget Sound strains of *H. akashiwo* using a recently developed in vitro microplate-based ichthyotoxicity assay using the rainbow trout cell line RTgill-W1 (Dorantes-Aranda *et al.* 2011).

Materials and Methods

Culturing

Heterosigma akashiwo strains NWFSC-503, -512, and -513 were isolated from two locations in Puget Sound; Clam Bay (July 1990), North Bay (August 2009), and Clam Bay (June 2010), respectively. Non-axenic, unialgal batch cultures were grown in filtered-sterilized (0.2-µm GPWP04700; Millipore) seawater, salinity of 30, and enriched according to Berges *et al.* 2001 (Corrigendum 2004), with modifications to the ESAW enrichment stocks as outlined by Auro and Cochlan (2013). Copper, as $CuSO_4 \cdot 5H_2O$, and selenium, as Na_2SeO_3, were prepared as separate stock solutions, and added to the medium to achieve final concentrations of 3.9 x 10^{-9} M and 5.8 x 10^{-9} M, respectively. Silicic acid was not added to the medium. Nitrate ($NaNO_3$) and phosphate (Na_2HPO_4) concentrations were reduced from the standard ESAW formulation, with nutrients added to achieve an initial medium concentration of 80 µM and 10 µM, respectively. Cultures were inoculated into 2.5-L borosilicate Erlenmeyer flasks at an initial cell density of 5,000 cells mL^{-1} and grown in a temperature-controlled environmental chamber (Forma Scientific) maintained at 14 °C (± 1.0 ºC) and illuminated at a photosynthetic photon flux density (PPFD) of. 80-100 µmol photons $\cdot m^{-2} \cdot s^{-1}$ on a 14 h:10 h, light: dark cycle. Cell abundances were determined in quadruplicate by light microscopy using Sedgewick-Rafter counting chambers.

Subsamples were collected aseptically for nutrients, cell abundance, and gill cell viability assays every 2-5 days for ≥ 30 days in culture to quantify the toxicity during both the nutrient-replete exponential growth phase, and the nutrient-depleted stationary phase of growth. Unfiltered samples were collected in pre-cleaned polypropylene tubes, and analyzed for nitrate plus nitrite (NO_3^- + NO_2^-; hereafter referred to as nitrate), ortho-phosphate, and silicic acid with a Lachat QuikChem 8000 Flow Injection Analysis system using standard colorimetric techniques (Smith and Bogren 2001; Knepel and Bogren 2002; Wolters 2002, respectively).

Preparation of *Heterosigma akashiwo* extracts

H. akashiwo cells were harvested and concentrated by centrifugation at 2,000 g for 10 min. The culture filtrate was gently discarded and the cells resuspended in L-15/ex medium to a final density of 200,000 cells mL^{-1} and then frozen at -20ºC to lyse cells. The frozen suspension was thawed within 7 days, inspected by light microscopy to ensure complete cell lyses, and assayed within 24 h. Cell preparations were vortexed upon thawing (to assist in lysis), resuspended, centrifuged at 3,500 g for 10 min, and held at 4ºC until assayed. The resultant supernatant was harvested and employed for the gill cell assay (GCA) experiments.

Cell line: origin, maintenance and culturing

The permanent epithelial cell line RTgill-W1, initiated from the gill filaments of rainbow trout *Oncorhynchus mykiss* (Bols *et al.* 1994), was obtained from the American Type Culture Collection (ATCC2523). The cells were maintained at 19 ± 1.0 ºC in the dark, and grown in 75-cm^2 high-quality, polystyrene flat bottom flasks (Falcon) in Leibovitz's L-15 medium (ATCC 30-2008), supplemented with 10% (v/v) fetal bovine serum (FBS, HyClone SH30071.02), and an antibiotic-antimycotic solution (15240-096, Gibco). A Trypsin - EDTA solution (59428C, SAFC Biosciences) was used to detach and subculture the cells every 10-14 days at a ratio of 1:3 with medium renewal every 3-4 days. Cells were counted using a haemocytometer, adjusted to a concentration of 10^5 cells mL^1 and seeded in quadruplicate onto 96-well, flat-bottom microplates (353072, Falcon Becton) at 100 µL per well. This procedure was carried out at least 48 h before the experiments in order to achieve a confluent monolayer

of the cells. Thirty-six hours after seeding the cells, the L-15 medium was discarded, and replaced with L-15/ex (Dayeh *et al.* 2005). After a further 12-36 h, the gill cells were rinsed with PBS and 200 µL of *H. akashiwo* extract (see below) in L-15/ex was added to each well (Schirmer *et al.* 1997). Viability was assessed after 4 h incubation of *H. akashiwo* extract with gill cells at 19°C in the dark. The methods described here are a modification of those described in Dorantes-Aranda *et al.* (2011).

Cell viability assays

Cell viability was determined with the indicator dye AlamarBlue (DAL1025, Invitrogen) (Pagé *et al.* 1993). Once exposure was completed, the exposure medium was discarded and the gill cells rinsed with phosphate buffer saline (PBS). L-15/ex containing 5% (v/v) AlamarBlue was subsequently added to all wells (100 ml per well), and cells were incubated for 2 h in the dark (Dayeh *et al.* 2005). The fluorescence of AlamarBlue was detected using excitation and emission filters of 540 and 600 nm, respectively, using a microplate reader (BioTek FLx800). The viability results are expressed as percentage of the readings compared to the controls (% of control, e.g., gill cells without *H. akashiwo* extract), and are reported for each sampling point as the mean and standard deviation (SD) of quadruplicate determinations.

Results and Discussion

We investigated the influence of different phases of cellular growth on the toxicity of three isolates of *H. akashiwo* isolated from the southern portion of the Salish Sea − Puget Sound, Washington. Toxicity was determined using an in vitro assay that directly exposes Rainbow trout gill cells (GCA) to the phytoplankton cells grown previously in batch cultures over ≥ 30 days in enriched seawater media at 14 °C − a relatively low temperature expected in situ for the spring season of the study region. The specific growth rates for the three Puget Sound isolates averaged 0.4-0.45 d^{-1} during the nutrient-replete, exponential growth phase (days 2-10), and are less than the growth rates reported for other *H. akashiwo* strains isolated elsewhere including Japan, Spain, New Zealand and the east coast of the USA, which range from 0.7 to 1.4 d^{-1} (see Herndon and Cochlan 2007). However, they are very similar to the rates (0.4-0.7 d^{-1}) reported for four other Salish Sea strains grown at a salinity of 30 and temperature of 15 °C (Strom *et al*. 2013), and appear to reflect the relatively slower growth rate of Salish Sea isolates when grown under laboratory conditions.

The exhaustion of nitrate in the medium employed in these experiments induced the transformation from exponential to stationary phase of growth in the three batch cultures of these isolates (Figure 1). External supplies of phosphate were still available at concentrations (≥ 3 µM) considered saturating for uptake, as were the ambient concentrations of silicic acid (10-20 µM) at the start of the stationary period and throughout the experimental duration. Micronutrients and vitamins were added in excess, and would not be expected to limit cell growth in cultures of such low density.

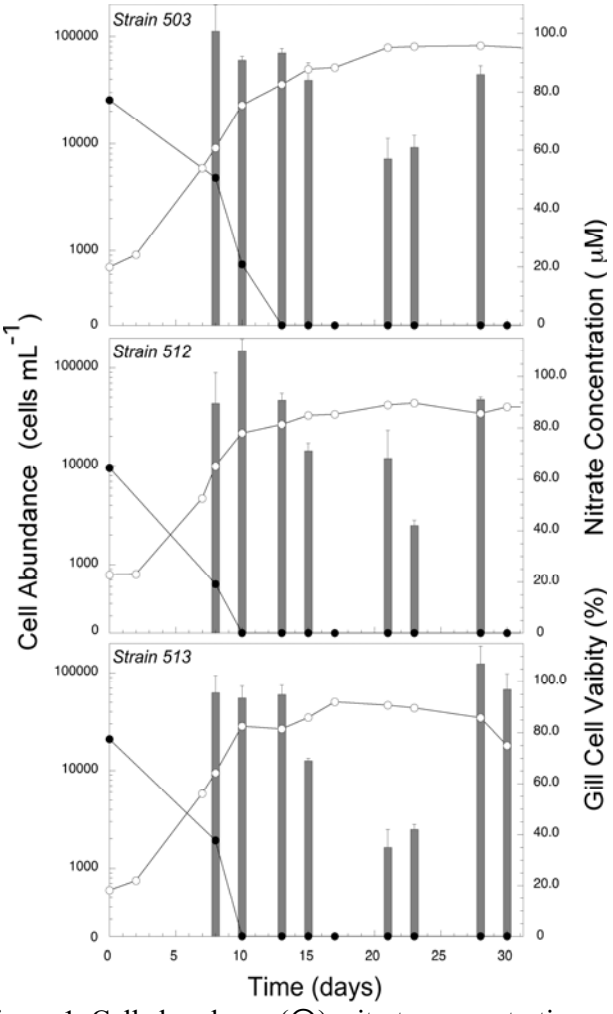

Figure 1. Cell abundance (◯), nitrate concentration (●), and gill cell viability (bars) as a function of time for strains NWFSC-503, -512 and -513. Error bars are ± 1 SD (n = 4) of replicate determinations.

The relative toxicity of the three isolates displayed a uniform pattern as a function of cellular growth phase and nutrient sufficiency. During the exponential and early stationary phases the cells were not toxic, and GCA viability averaged 95% of the controls, where GCA preparations were not exposed to *H. akashiwo* cells. After a minimum of 5 days of nitrate exhaustion in the medium, during which time phytoplankton cell accumulation was minimal, GCA viability initially declined, and further decreased to an average of 59, 55 and 39% of control values during mid-stationary growth phase in strains 503, 512 and 513, respectively. Finally, *Heterosigma* cells became non-toxic during late stationary phase after a total of 15-18 days of nitrate depletion. We speculate this loss of toxicity is the result of degraded membrane integrity and the leakage of the putative toxin(s) from the *Heterosigma* cells or its association with cellular membranes. Neither the identification of a putative toxin nor determination of its concentrations were attempted in this study, however the toxicity assay employed here used a constant number of *H. akashiwo* cells (1 mL extract of 200,000 cells per treatment). Consequently, the viability results reported here can be considered equivalent to toxin concentration normalized to cell density; commonly referred to as either cell toxin quota or cellular toxin concentration. Variation in toxin production as a result of physiological changes associated with growth stage or nutrient availability of batch cultures, termed 'growth stage variability' by Anderson *et al.* (1990) has been documented in other harmful algal species (e.g., Granéli and Flynn 2006), but the present study is the first to report such toxin variability in this cosmopolitan raphidophyte impacting the Salish Sea and elsewhere. Our results also suggest that NWFSC-503 is the least toxic of the three strains tested, which may be due to its extended period under cultivation as it was isolated 19 and 20 years before NWFC-512 and -513, respectively.

Acknowledgements

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Efficacy of *Microcystis aeruginosa* removal in deionized and brackish water

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Abstract

In recent years, harmful algal blooms have become a significant global concern due to their increasing occurrence and negative environmental impact. The Chesapeake Bay represents a model for estuaries facing serious ecological problems due to the growing frequency of HABs. *Microcystis aeruginosa* is a prominent bloom former in the Bay. This cyanobacterium forms high biomass (and occasionally toxic) blooms in tidal-fresh, low salinity regions of the Bay and its upper tributaries. In this study, we evaluate the ability of 88 different sediment-chitosan mixtures to remove *M. aeruginosa* cells from suspension in the laboratory. Nine local sediments and two commercially available clays were tested across a range of sediment concentrations in combination with varying amounts of diluted chitosan to determine which mixtures effectively removed cells from the water column. Flocculation mixtures were tested for cell removal efficiency in deionized water and the most effective mixtures were then tested in filtered creek water. Results showed that relatively low concentrations (0.25 gL^{-1}) of local sediments with 5 mgL^{-1} or 2.5 mgL^{-1} chitosan could effectively remove cells from the water column (≥90% removal) in less than one week. Hence, flocculation using local sediments may be a promising method for mitigating estuarine *M. aeruginosa* HABs.

Keywords: Chitosan; clay flocculation; cyanobacterial bloom; *Microcystis aeruginosa*

Introduction

Clay flocculation has proven effective in mitigating many freshwater algal blooms with the cells' affinity for sediment particles varying across algal species (Avinmelech *et al*. 1982). Thus far, clay flocculation has been used in a number of countries, including nations of East Asia, Australia, Sweden, and the United States (Sengco and Anderson 2004) to treat and prevent HABs. Estuaries, like the Chesapeake Bay, are characterized by high concentrations of suspended sediments, stratification of the water column throughout much of the year, and often incur HAB events due to increased nutrient loading from urban and farm runoff. While many studies have focused on HAB flocculation in lakes (Zou *et al*. 2006; Wang *et al*. 2012), no studies exist on mitigating estuarine HABs using local sediment flocculation. One global bloom former that is especially pervasive in the Chesapeake Bay is *Microcystis aeruginosa.* This particular cyanobacterium commonly forms high biomass blooms in low turbulence, low salinity waters, from late spring to early fall (Jöhnk *et al*. 2008) and thrives in the upper regions of the Bay where the water is still and relatively fresh. Previous studies have found that modified local sediments (Zou *et al.* 2006) and local sands (Pan *et al*. 2011) can effectively remove algal cells from suspension. For this study, we collected polymineralic sediments from the Chesapeake Bay watershed area and assessed their ability to flocculate *M. aeruginosa* cells. Local sediments were chosen in order to minimize the environmental impact of sediment introduction as well as the cost associated with processing and transporting sediments (Cho *et al*. 2012). In previous studies, the addition of a flocculant significantly reduced the amount of sediment needed for effective cell removal (Zou *et al*. 2006; Liu *et al*. 2010; Pan *et al*. 2011). We used chitosan as a flocculant in this study because it is a derivative of one of the most common natural biopolymers chitin (Ahmad *et al.* 2011). Our initial experiments were designed to provide baseline

information regarding the mineral and charge properties of the local sediments and the removal efficiencies of the sediment/chitosan mixtures in deionized water (DI). It was thought that charge characteristics of native sediments for mixtures that removed ≥90% cells in <1 week in DI might provide an indication of potential removal efficiencies in Chesapeake Bay estuarine creek water. Our ultimate goal is to develop an easily applicable, inexpensive technique for routine use in bloom mitigation in regional waters.

Methods

M. aeruginosa cultures (UTEX 2667) were obtained from the University of Texas at Austin and grown in BG-11 medium (Pan et al. 2006; Zou et al. 2006) that was prepared with deionized water (DI). Stock cultures were maintained at 22°C under a 14/10 light/dark cycle throughout the study. Cell growth was tracked with *in vivo* fluorescence (IVF) determined using a model 10-005R Turner Designs Fluorometer. A linear regression between IVF and cell concentration (determined with a haemocytometer) was established for estimating cell abundances.

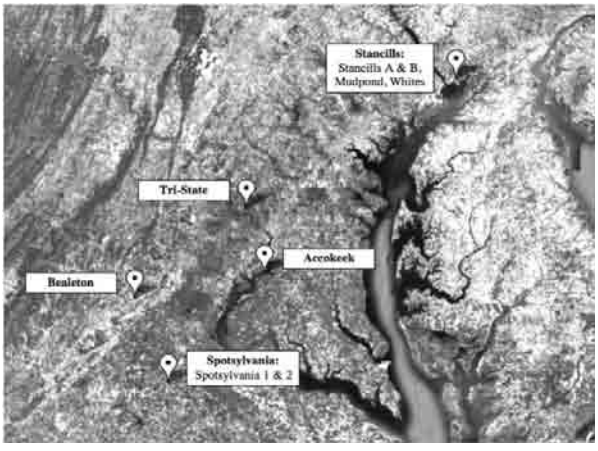

Fig. 1. Locations of sediment collection sites in the Chesapeake Bay watershed.

Processed clays were obtained from two commercial suppliers: kaolin from Thiele Kaolin Company (Sandersville, GA, USA) and montmorillonite from Eytons' Earth (Las Vegas, NV, USA). Nine local sediments were obtained from five locations within the Chesapeake Bay watershed (Fig. 1). The mineralogy of the local sediments was determined by X-ray diffraction of oriented slides, which was prepared by dispersing the sediment in water, allowing particles to settle onto a glass slide, and then air drying under a heat lamp. Spectra were collected using a Bruker D8 diffractometer using Cu Kα radiation and standard determinative techniques for ≥1 h at 550ºC as described in Poppe et al. (2001).

The mineral composition determined by X-ray diffraction (XRD) varied across the nine local sediments (Table 1). Qualitative abundances were based on the relative intensities of XRD peaks, recorded as very strong (V Str), strong (Str), moderate (Mod), or weak (Weak). The columns from left to right show minerals with increasing cation exchange capacity (CEC). Kaolin and montmorillonite, were assumed to be monomineralic, not confirmed by XRD.

Table 1. Mineralogical composition of local sediments based upon X-ray diffractometry (XRD).

	Quartz	Kaolinite	Illite	Smectite	Vermiculite
ccokeek	V Str	Mod	Str		Mod
ealeton	V Str			Str	
pots. 1	V Str	Str	Mod		Mod
pots. 2	V Str	Str	Mod		
tancills A	Mod	Str	Mod		
tancills B	Mod	Str	Mod		
tancills MP	Mod	V Str	Mod		
tancills W	Mod	Str	Mod		
ristate		Str	Str	Weak	Str

Prior to flocculation experiments, 10 and 5 gL^{-1} sediment slurries were made in DI and used within one day of preparation. A 10 gL^{-1} chitosan solution was made by combining 0.5 g dry chitosan with 0.05 L 1% HCl and mixing until dissolved. The 10 gL^{-1} chitosan stock solution was used within six months and stored at room temperature protected from light. For each of the 11 sediments, eight experimental mixtures were tested, yielding a total of 88 different sediment-chitosan mixtures. The eight mixtures include 0.25 gL-1 sediment supplemented with 0, 2.5, 5, and 25 mgL^{-1} chitosan and 0.5 gL^{-1} sediment supplemented with 0, 5, 10, and 50 mgL^{-1} chitosan. These concentrations correspond to 100:1, 50:1, and 10:1 ratios of sediment to chitosan, respectively, and refer to the final concentration of sediment and chitosan after the mixture was added to the algal culture. Flocculation experiments were conducted in 50 mL tubes with an *M. aeruginosa* density of 10^7 cells mL^{-1}. Using a pipette, 2 mL of each flocculation mixture was added to 38 mL of

suspended *M. aeruginosa* in DI. Each flocculation mixture was assayed in triplicate and tubes were not disturbed post-slurry addition. For the controls, 2 mL of DI was added instead of a flocculation mixture. IVF measurements were taken immediately upon slurry addition after which the tubes were placed in a rack to allow flocculation to proceed. Subsequent IVF readings were taken at the same time each day (+/- 2 h) for seven days. IVF measurements across each triplicate were averaged and used for analysis.

Maximum percentage of cells removed was used to assess the removal efficiencies of the flocculation mixtures. This was calculated by determining the number of cells removed at each assayed time using the aforementioned regression relating IVF to cell concentration. We subtracted the number of cells in the experimental conditions from the number of cells in the control conditions. We then divided this value by the number of cells in the control conditions and multiplied the resulting value by 100 to get the % Cell Removal ([(Cont-Exp)/ Cont]*100). Maximum cell removal was reported as the greatest percentage of cells removed at any point during the weeklong observation period. Time to 50% cell removal was calculated by graphing cell removal over time and extrapolating the time at which 50% of the *M. aeruginosa* cells had been removed from the water column based on a line of best fit. This was done independently for each triplicate and then averaged. All results for maximum percent cell removal and time to 50% cell removal are reported as the average of the triplicates +/- SE (standard error). Results were analyzed with PASW Statistics 18 software, using one-way ANOVA. Levene's statistic was used to assess variance. Games-Howell *post-hoc* tests were used when variances were unequal and Tukey's *post-hoc* tests were used when variances were equal.

Results and Discussion

Of the 88 flocculation mixtures tested for removal efficiency of *M. aeruginosa* in DI, 11 mixtures showed ≥90% cell removal in <1 week (Table 2). While these 11 mixtures all showed high maximum percent removal, they differed in removal speed. The average time to 50% cell removal for those mixtures was 30.5 h (+/- 28.7 h). Sediment concentration did not significantly affect mixture efficiency in DI ($p = 0.263$), but chitosan-modified mixtures removed cells significantly better than mixtures that contained sediment alone ($p = 0.001$) (Fig. 2). No sediment mixtures showed ≥50% cell removal without the addition of chitosan. Over the eight sediment-chitosan combinations tested across all 11 sediments, mixtures with a 50:1 ratio of sediment to chitosan were the most effective (Fig. 3). Higher chitosan concentrations decreased removal efficiency, which has also been observed in other studies (Ahmad *et al*. 2011). Several flocculation mixtures, such as those composed of the suite of Stancills sediments (Table 2), could effectively and rapidly flocculate suspended *M. aeruginosa* cells in DI water with even lower chitosan concentrations (1:100).

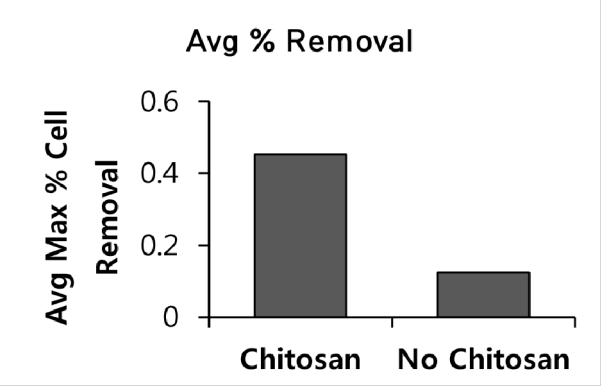

Fig. 2. Average maximum % cell removal in <1 week using sediment mixtures with (n=66) and without chitosan (n=22). Error bars represent standard deviation of the mean.

Table 2. Flocculation mixtures that removed ≥90% cells in <1 week in DI with laboratory cultured *M. aeruginosa* (UTEX 2667). 10:1, 50:1, and 100:1 refer to the concentration of sediment to chitosan in each flocculation mixture.

Flocculation Mixture	% Max Cell Removal	Time to 50% Cell Removal (h)
Bealeton 0.25 gL^{-1} 10:1	100 (+/- 0)	10.6 (+/- 0.5)
Tristate 0.5 gL^{-1} 50:1	100 (+/- 1)	89.0 (+/- 14.5)
Stancills B 0.25 gL^{-1} 100:1	100 (+/- 1)	23.4 (+/- 5.8)
Stancills A 0.25 gL^{-1} 100:1	100 (+/- 1)	39.4 (+/- 0.4)
Stancills MP 0.5 gL^{-1} 100:1	99 (+/- 1)	2.2 (+/- 1.3)
Stancills MP 0.5 gL^{-1} 50:1	98 (+/- 1)	4.6 (+/- 1.0)
Stancills B 0.25 gL^{-1} 50:1	98 (+/- 2)	76.0 (+/- 4.5)
Kaolin 0.25 gL^{-1} 10:1	97 (+/- 3)	36.2 (+/- 1.5)
Stancills W 0.5 gL^{-1} 100:1	96 (+/- 2)	13.7 (+/- 0.7)
Stancills A 0.25 gL^{-1} 50:1	95 (+/- 1)	26.7 (+/- 7.4)
Stancills MP 0.25 gL^{-1} 50:1	94 (+/- 1)	14.2 (+/- 1.9)

This study demonstrated that sediment-chitosan flocculation was successful in the removal of *M. aeruginosa* cells along a range of sediment and chitosan concentrations and sediment to chitosan ratios of 1:50 in the flocculation mixtures were optimal for most of the sediments tested (Fig. 3).

The same protocol was followed to test the effectiveness of the 11 flocculation mixtures that removed ≥90% cells in <1 week in DI (Table 2), but using laboratory cultured *M. aeruginosa* suspensions (UTEX 2667) in filtered and autoclaved water from Mattawoman Creek, MD, a tidal, brackish (<0.5 ppt) tributary of the Chesapeake Bay that routinely experiences *M. aeruginosa* blooms. Bealeton 0.25 gL^{-1} with 10:1 chitosan, Tristate 0.5 gL^{-1} with 50:1 chitosan, and Kaolin 0.25 gL^{-1} with 10:1 chitosan showed similar high cell removal in filtered creek water as in DI and removed ≥90% cells in <1 week. In contrast, Stancills sediments (A, B, Whites, and Mudpond) were significantly less effective at removing *M. aeruginosa* cells in the filtered creek water than they were in DI ($p < 0.000$). Differences in removal efficiency between ion free and brackish conditions may be attributed to charge alterations in the clay lattice through solvent-clay reactions, which alter the substituted clay-chitosan interactions and flocculation results.

These results suggest that flocculation using local sediments is potentially an effective mitigation method for treating *M. aeruginosa* blooms, warranting further study in estuarine mesocosms (Pan *et al*. 2006), with the goal of developing a field technique for routine intervention (Wang *et al*. 2012). The results of this study demonstrate that local sediments could remove *M. aeruginosa* cells as effectively as the commercial clays ($p = 0.510$). Therefore, local sediments should be preferentially used in flocculation processes due to their effectiveness relative to commercial sources, lower cost, likely lower environmental impact as 'native' sediments, and the increased 'comfort' of regional citizens when given the choice between local and foreign materials (Cho *et al*. 2012).

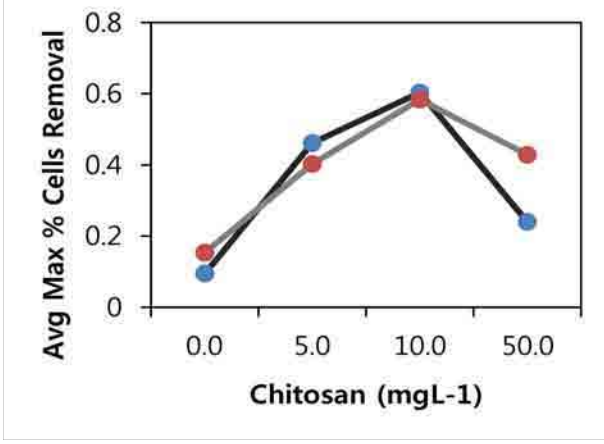

Fig. 3. Effects of sediment-chitosan mixture on max % cell removal in <1 week, averaged across all 11 sediments in DI water. Light grey = 0.25 gL^{-1} sediment mixtures; dark grey = 0.5 gL^{-1} sediment mixtures (error bars = SE).

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The effects of light, temperature and nutrient stress on laboratory cultures of *Heterosigma akashiwo* from Puget Sound, Washington, USA

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Abstract

The raphidophycean flagellate, *Heterosigma akashiwo*, (Y. Hada) Y. Hada ex Y. Hara et M. Chihara is associated with extensive finfish mortality in commercial aquaculture operations in Puget Sound, Washington, and may also affect wild nekton in the Salish Sea waters of British Columbia and Washington. The environmental factors thought to influence the growth of four *H. akashiwo* isolates from Puget Sound were examined in laboratory batch cultures to evaluate inter-strain variation in specific growth rates and the ratio of nutrient (N:P) draw-down as a function of photosynthetic photo flux density (PPFD), temperature and nutrient limitation. Long-term culturing under conditions dissimilar to those expected in situ results in measurable differences in the response to elevated PPFD; photoinhibition was absent in cultures grown at 20°C after exposure to 1000 µmol photons m^{-2} s^{-1} (μ = 0.77 d^{-1}), whereas at 15°C photoinhibition occurs at 428 µmol photons m^{-2} s^{-1} (μ = 0.57 d^{-1}). Temperature affects the uptake ratio of N and P. The N:P draw-down ratio of cultures grown at 20°C were greater than the N:P draw-down ratios at 15°C, regardless of the ambient N:P ratio in the media. These findings re-enforce the need for appropriate growth conditions (e.g., isolate duration, photoadaptation and medium N:P ratio) in relation to experimental objectives.

Keywords: *Heterosigma akashiwo*, nutrient limitation, photoadaptation, isolate variability

Introduction

Laboratory studies provide researchers with a controlled environment to evaluate the importance of factors thought to regulate growth and toxicity of phytoplankton species that form harmful algal blooms (HABs). However, long-term laboratory maintenance of phytoplankton isolates grown under environmental conditions dissimilar to those expected in the natural environment brings into question the possibility that significant 'physiological drift' may have occurred since the original isolation. To support an on-going study of the marine raphidophyte, *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara et M. Chihara in the Salish Sea, a preliminary study was conducted to examine four different isolates of *H. akashiwo* from Clam Bay, Washington, USA, to establish a baseline for future research. The objectives of the study presented here are as follows:
1. Determine the degree of variability among isolates of *H. akashiwo*, as a function of time maintained in culture since isolation from the same geographical region, in response to light and temperature changes.
2. Determine if *H. akashiwo* cells from the Puget Sound region display a similar lack of photo-inhibitory growth response at elevated photosynthetic photon flux density (*ca.* 1000 µmol photons m^{-2} s^{-1}), as observed for natural populations from Spain (Butrón *et al.* 2012).
3. Quantify the effects of N and P limitation on specific growth rates and the N:P draw-down ratios of isolates grown at two environmentally relevant temperatures (15 and 20°C).

Methods

The four isolates of *H. akashiwo* used in this study were collected from Clam Bay, WA, (across from the City of Seattle in central Puget Sound) at different collection dates (Table 1), and maintained under non-axenic conditions. For the experiments presented here, unialgal batch cultures were grown in 0.2-µm filtered (Polycap 150 TC; Whatman) seawater, collected from Monterey Bay, CA, USA. Seawater was enriched with modified ESAW (Berges *et al.* 2001; Harrison *et al.* 1980) where both N and P concentrations were reduced (50 and 5 µM, respectively), to prevent inorganic carbon limitation

due to biological activity (e.g., Howard *et al.* 2007). Cultures were grown within a Sanyo Versatile Environmental Test Chamber, capable of maintaining ambient temperature to ± 0.5°C of the desired experimental temperature. All experiments were conducted under a 12 h light: 12 h dark cycle. The average photosynthetic photon flux density (PPFD) for each treatment was measured using a 4π collector immersed in each culture vessel, and read with a Biospherical Instruments QSL-100 quantum scalar irradiance meter. The desired PPFDs were obtained by using a variety of grades of neutral density plastic film (LEE Filters), which evenly reduces the incident PPFD produced by the fluorescent bulbs of the incubator chamber. Prior to any culturing, all necessary glassware, plasticware and equipment was cleaned with 10% HCl acid (v/v) before autoclaving at 121°C for 15 minutes. Algal biomass was assessed daily using *in vivo* fluorescence (Turner Designs 10-AU), and/or by cell abundance determined microscopically using a Sedgewick Rafter counting chamber (#1801-G20; Wildlife Supply Company).

Table1: Collection dates and locations for the four different NWFSC isolates used.

Isolate	Location	Date of isolation	Isolated by
503	Clam Bay, WA	July 4, 1990	Dr. Rita Horner
513	Clam Bay, WA	June 16, 2010	Mr. Brian Bill
514	Clam Bay, WA	June 16, 2010	Mr. Brian Bill
517	Clam Bay, WA	June 23, 2010	Mr. Brian Bill

Prior to enumeration, cells were persevered with 5% Lugol's acid solution (Sigma Aldrich), and allowed to settle for 5 minutes before counting. Care was taken to ensure preserved samples did not sit longer then 30 minutes, in order to prevent cell lysis prior to enumeration. Specific cell growth rates were calculated from a least-squares linear regression analysis of the exponential phase of cell growth, determined from plots of the natural log of cell biomass (measured by either *in vivo* fluorescence or cell abundance) versus time.

Results and Discussion

Growth rate variability as a function of PPFD and temperature.

Three *H. akashiwo* isolates (NWFSC-503, NWFSC-514 and NWFSC-517) were used to determine the degree of 'physiological drift' that could have occurred during long-term maintenance since their original isolation. Isolate NWFSC-503 was collected *ca.* 20 years prior to either NWFSC-514 or NWFSC-517, whereas NWFSC-514 and NWFSC-517 were collected only 7 days apart.

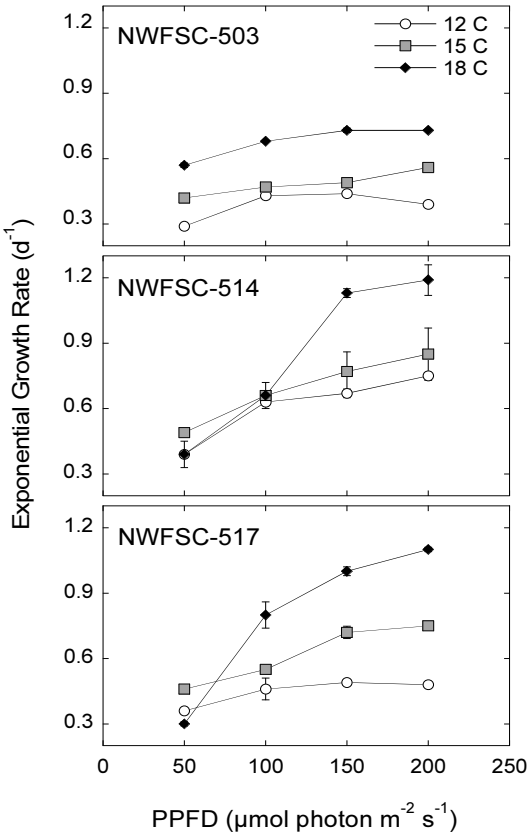

Fig. 1. Mean exponential growth rate (n=2) for NWFSC-503, -514 and -517 as a function of temperature. Error bars indicate the range of duplicates; no error bars indicate that errors are smaller than symbol width. Growth rates were determined from daily *in vivo* fluorescence measurements of cultures.

Each isolate was exposed to three temperatures (12, 15 and 18°C) at four different PPFDs that ranged from 50 to 200 µmol photons m^{-2} s^{-1} for over 8 generations. The temperatures employed here represent the annual variation in temperature expected within the Salish Sea (the estuarine system comprising Puget Sound, the Strait of Georgia, and the Strait of Juan de Fuca on the North American west coast). The lowest temperature (12°C) represents the overwintering temperature during winter months, 15°C is water temperature prior to bloom formation, and 18°C is the water temperature

during blooming periods (Rensel *et al.* 2010). Each isolate was pre-acclimated to the respective temperature and 100 µmol photons m^{-2} s^{-1} for 10 days prior to beginning the experiment. All three isolates displayed no photo-inhibition at PPFDs up to 200 µmol photons m^{-2} s^{-1}, and the specific growth rates increased as a function of increasing PPFD and temperature (Figure 1). At low PPFD (50 µmol photons m^{-2} s^{-1}), the specific growth rates were similar for all three isolates ranging from 0.6 to 0.8 d^{-1}, whereas at the higher PPFD (150-200 µmol photons m^{-2} s^{-1}) both NWFSC-514 and NWFSC-517 grew faster (μ = 1.2 d^{-1} and 1.1 d^{-1} respectively), than NWFSC-503 (μ = 0.7 d^{-1}). For each isolate, growth at 12°C was slowest, and growth rates increased as a function of temperature. These results suggests that cell growth below 18°C is sub-optimal, and temperature is likely limiting enzyme activity during the dark reactions of photosynthesis (Davison 1991). They also highlight the greater degree of 'physiological drift' that has occurred during the extensive culturing period for isolate NWFSC-503 compared to the more recently collected isolates: NWFSC-514 and NWFSC-517. Although no genetic analyses were conducted to identify potential differences between the isolates, the drastically different physiological response of NWFSC-503 to higher PPFDs and temperatures than either of the more recently collected isolates (NWFSC-514 and NWFSC-517) suggests it possesses a different genetically-driven adaptive ability to PPFD and temperature due to its long-term maintenance at suboptimal conditions for growth as a library culture (Kirk 1994). It is important to note that none of the isolates were photoacclimated prior to beginning the experiments, and it is not clear if NWFSC-503 would have behaved similarly to NWFSC-514 and NWFSC-517 if allowed to properly acclimate to the environmental conditions prior to experimentation.

Photoacclimation at high PPFD.

Research conducted by Butrón *et al*. (2012) found that natural populations of *H. akashiwo* from the Nervión-Ibaizabal estuary, Spain, displayed no photo-inhibitory effects up to c*a.* 1200 µmol photons m^{-2} s^{-1}. Here we conducted a study to determine if a single Clam Bay isolate demonstrated the same lack of photoinhibitory response even though the test isolate was maintained under two years of environmentally-irrelevant conditions of reduced PPFD and temperature. Isolate NWFSC-513 was pre-acclimated to either 15 and 20°C and then grown under seven different PPFDs ranging from 50-1000 µmol photons m^{-2} s^{-1} without pre-acclimation to the various light levels. Results using these cells indicate that photo-inhibition occurred for both 15 and 20°C treatments, with photoinhibition occurring roughly after 300 µmol photons m^{-2} s^{-1} in the 20°C treatment and after 150 µmol photons m^{-2} s^{-1} in the 15°C treatment (Figure 2A). The experiment was immediately repeated with photoacclimated cells for each PPFD, with all other conditions remaining the same. Similar to the trend observed with non-photoacclimated cells, the 20°C treatment produced faster growth rates than 15°C, but no photoinhibition was observed at 20°C and only reduced photo-inhibition at 15°C (Figure 2B).

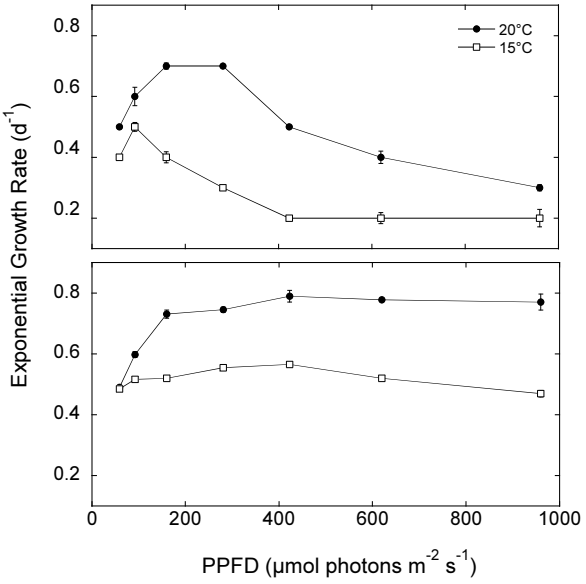

Fig. 2. Growth rates of NWFSC-513 determined by *in vivo* fluorescence; values represent the mean ± the range of duplicates (n=2). (A) Cells for each PPFD treatment were acclimated to the respective temperatures, but not PPFD. (B) Cells were acclimated to both temperature and PPFD.

These findings indicate that *H. akashiwo* isolated from the Clam Bay region have similar photoadaptive abilities as the isolates from Nervión-Ibaizabal estuary, since the effects of photoinhibition were either prevented or reduced as a function of temperature. Equally important to these findings is the ability of *H. akashiwo* to acclimate in only three generations of growth to PPFDs that were substantially different from those experienced as library maintenance cultures. This may be explained

by the fact that chloroplast replication in *Heterosigma* is separate, but synchronized to cell division (Satoh *et al.* 1987).

N:P draw-down rates.

Cultures of isolate NWFSC-513 were grown in triplicate at 15 and 20°C under previously determined saturating PPFD conditions (*ca.* 200 µmol photons m^{-1} s^{-1}) to examine the effect of the medium N:P ratio and to determine whether N or P limitation influences the measured N:P draw-down rates. Cultures were grown under nutrient-replete conditions with the induction of stationary growth phase as a result of either N or P limitation. A previously determined simultaneous draw-down ratio of approximately 10:1 was used as a template for establishing initial ambient concentrations of N and P, with nutrient limitation being achieved by the enrichment of an additional 25% of the non-limiting nutrient.

Results indicate that during exponential growth when both N and P were saturating for growth, only temperature affected the draw-down ratio regardless of the ambient N:P ratio in the media. At 20°C, the N:P draw-down ratio under potential N-limiting conditions averaged 15.6 (SD = 0.54) and under P-limitation it averaged 17.4 (SD = 1.17), with growth rates of μ = 0.8 and 0.7 d^{-1}, respectively. At 15°C the N:P draw-down ratio under the potentially N-limiting treatment was 11.2 (SD = 1.13) and under P-limitation it averaged 10.9 (SD = 2.52), with specific growth rates of 0.5 and 0.4 d^{-1}, respectively. However, when both the exponential and stationary portions of the growth curve are considered for all treatments, we find that at 20°C, the N:P drawdown ratio was 13.7 (SD = 0.15) under N limitation, and 59.8 (SD = 0.98) under P limitation. At 15°C, the N-limited cultures attained an average N:P draw-down ratio of 13.6 (SD = 0.45) and the P-limited cultures averaged 31.9 (SD =1.54). In summary, the N:P ratios in the media when concentrations were saturating for uptake/growth affected neither specific growth rates nor draw-down rates attained within each temperature examined. However, once one of the nutrients was exhausted due to phytoplankton uptake and stationary growth began, there was a difference in the overall N:P draw-down ratios with N-limitation resulting in lower ratios, and P-limitation resulting in considerably higher ratios; this was measured at both tested temperatures. While the N:P draw-down ratios for the N-limiting treatments at both temperatures were essentially the same, there was a substantial difference in the overall draw-down ratio for the P-limiting cultures as a function of temperature. Here the N:P draw-down ratios during the exponential, nutrient-replete portion of the experiments were greater at the higher temperature.

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Thalassorheology: cultures of harmful algae and bacteria increase or reduce laminar-flow viscosity depending on length scale

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Abstract
Microalgae and bacteria are known to impart the following rheological changes to their medium: 1) increased viscosity and elasticity in laminar flow; 2) decreased viscosity (drag reduction) in turbulent flow. Here we show that they can also impart: 3) decreased viscosity in laminar flow dependent on the length scale of tube radius. Possible mechanisms for this are discussed. We illustrate these concepts with measurements on laboratory cultures of the diatom *Skeletonema costatum*, dinoflagellates *Karenia mikimotoi* and *Alexandrium catenella*, and bacterium *Escherichia coli*.

Keywords: Rheology, Viscosity, Drag reduction, Laminar flow, Harmful algae, Bacteria.

Introduction.
Thalassorheology, the viscosity and elasticity of seawater, has been reviewed by Jenkinson and Sun (2010). Phytoplankton and bacterial exopolymeric substances (EPS) can: 1) decrease turbulent viscosity (Hoyt & Soli, 1965); 2) add elasticity and excess viscosity in laminar flow. Some HAB cultures can reduce flow in fish gills enough to kill them under typical HAB concentrations and conditions of gill flow (Jenkinson and Arzul, 2002). Both in algal and bacterial cultures as well as in the sea, EPS contribute excess polymeric viscosity η_E to the viscosity contributed by water and salt η_W, so that total viscosity, $\eta = \eta_W + \eta_E$. Furthermore, the value of η_E is related to shear rate generally negatively, and to phytoplankton concentration generally positively, for example in HABs (Jenkinson and Sun 2010). Sludge bacteria also produce aggregated EPS that increases resistance to flow (Spinosa and Lotito 2003), that was shown to have depended on length scale by Jenkinson *et al*. (2007), who suggested that η_E in the sea might likewise depend on length scale. A model to investigate possible control by phytoplankton EPS (PEPS) of pycnocline stability found that such control was highly dependent on the value of a length-scale-dependency exponent (Jenkinson and Sun 2011).
To validate this model experimentally, we investigated the viscosity of different phytoplankton cultures and one bacterial culture over a range of length scales and a range of shear rates. To do this we used capillary flow. During ICHA 14 we presented the methodology (Jenkinson and Sun 2012).

Material and Methods
Viscosity was determined by measuring flow rate as a function of hydrostatic pressure difference h and capillary radius using an Ostwald-Ubbelohde viscometer (OUV) (Jenkinson and Sun, 2012, Fig. 1). The OUV was modified in 4 ways: 1) Replacement of the single capillary tube by a modules containing batteries of capillary tubes of 5 internal radius r values, 0.35 0.5, 0.75, 1.05 and 1.5 mm; 2) A wide in-line tap at the bottom of the OUV inlet arm; 3) the connection of the two arms by a tube of internal radius1.5 mm, the yield stress tube (YST). During the flow the YST was kept closed, but after flow had stopped the tube was opened, to detect any length-scale-related yield stress; 4) the connection of each arm of the OUV by tubes to the two sides of a Honeywell USA DC010NDC4, high-sensitivity pressure probe, itself connected to a ADS 1062C oscilloscope (Atten, China), digitiser and a PC, to record change in differential pressure in real time (3000 data points over 60 s). The two arms of the OUP were rigid and kept rigorously vertical, room temperature was controlled, and cultures and reference liquids were allowed to equilibrate to room temperature before measurements. Table 1 shows details of the cultures measured.
Experimental procedure: Hydrostatic pressure, $P = h.$gravity, was calibrated against voltage output by the pressure probe by measuring h the height difference

between the menisci in each of the arms. All materials were temperature-equilibrated (+/- 0.5°C). Before use, each capillary module (CM) was rinsed thoroughly with tap water then with test or reference material, then inserted in-line in the viscometer. With the tap open, test or control liquid was gently added to the input arm of the viscometer and allowed to rinse through the apparatus including the CM, and homogenise the liquid in the viscometer. Using a syringe, excess liquid was withdrawn always from the output arm, thus avoiding reverse flow and any possible contamination of newly added liquid. After rinsing the viscometer, and making sure any bubbles had escaped, the tap was closed and further liquid was added to produce a value of P of 5 to 6 kPa. Data recording on the oscilloscope was activated and the tap was opened, allowing liquid to flow and P to decrease roughly exponentially towards the yield stress Y. After 45 s of flow, the YST was opened, and any reduction in P was conservatively assumed to represent the residual hold-up pressure Y due to yield stress in the culture (Spinosa and Lotito, 2003). As far as we are aware this is the first time that capacity to measure yield stress has been incorporated in an Ostwald or Ubbelohde capillary viscometer. Flow-curve measurements were replicated ~10 times and expressed in comparison with pure seawater (SW) or *Milli-Q* water, also replicated ~10 times. Flow measurements for each species were done on the same day or on two consecutive days, except in the case of *Alexandrium catenella*, in which 3 days elapsed between the trials. Files of data, digitised in ASCII by the recording oscilloscope, were transferred to a computer and processed by routines written in *Mathcad 14*. Shear rate at the wall was calculated as a function of both shear stress at the wall and length scale. The theory of capillary viscometers is given in more detail by Philippoff and Gaskins (1958).

Results

Hold-up pressure, which may be related to yield stress, or to wetting of the viscometer arms, showed no significant difference between cultures and reference water. Fig. 1 shows the total (laminar + turbulent) relative viscosity (RV) of different cultures relative to seawater (*Milli-Q* water in Fig. 1d), corrected for the small difference between the cultures and the seawater due to salinity. Standard deviation (SD) in η was everywhere greater in the low-P range (100 to 20 Pa) than in the high-P range (500 to 100 Pa). This higher SD reflects the lower relative precision in the pressure probe at low P, but may also include a higher contribution from zeroing error also at low P, as well as really greater variation in η_E. Fig. 1 shows RV vs r for cultures of the dinoflagellates *Karenia mikimotoi* and *A. catenella*, the diatom *Skeletonema costatum* and the bacterium *Escherichia coli*. At high P, *K. mikimotoi* showed RV significantly >1 at r values of 0.35 and 0.5 mm, but values close to 1 at r values from 0.75 to 1.5 mm. Both *A. catenella* and *S. costatum*, by contrast, showed high-P RV values >1 or close to 1 for all r values. *E. coli* also showed high-P RV values just above 1 at low r values, but close to 1 at r values from 0.75 to 1.5 mm. At low values of P, *K. mikimotoi* culture showed RV considerably greater than 1 at r values from 0.35 to 0.75 mm, but significantly reduced RV at an r value of 1.5 mm. *S. costatum* showed RV values close to 1 at r values from 0.35 to 0.75, but significantly and strongly decreased RV at r values of 1.05 and 1.5 mm. Rather similarly, *A. catenella* showed RV values <1 or close to 1 at all r values, (except for at 0.75 mm, where RV was >1, but not significantly so). Abundant encystment took place with sticking of cysts to the walls of the capillaries reducing their real radii during measurements made at notional r values from 0.75 to 1.5 mm, and may, by narrowing the capillaries, have led to RV being overestimated. *E. coli* culture showed RV not significantly different from 1, except at an r value of 1.5 mm, where it was markedly <1. In summary, for *E. coli* in the low-P range, for *S. costatum* in the high-P range and for *A. catenella* in both ranges of P, any relationship between RV and r was unclear. In contrast, for *K. mikimotoi* in both the high-P and the low-P ranges, for *S. costatum* in the low-P range, and for *E. coli* in the high-P range, the relationships between RV and r were negative.

Discussion

Increase in RV, i.e. drag increase, occurred relatively more in the smallest capillaries. We ascribe it to increase in molecular viscosity caused by phytoplankton EPS, coupled with the length-scale relationship already found in sludge EPS flowing in capillaries, as mentioned in the Introduction. Unexpectedly, however, in the wider capillaries we found considerable decrease in RV, i.e. drag reduction (DR), particularly in the low-P range.

Table 1. The cultures measured. Cell biovolumes are from values used by Ou *et al.* (2008) for *S. costatum* and *A. catenella*, from *Algaebase* (Guiry & Guiry, 2012) for *K. mikimotoi*, and from Lee and Fuhrman (1987) for *E. coli.*

Species	Concentration (cells mL^{-1})	Cell biovolume (μm^3)	Volume fraction (naked organism)
Skeletonema costatum (s.l.)	2.2×10^6	196	0.43%
Alexandrium catenella	3.3×10^3	14,130	0.047%
Karenia mikimotoi	6.3×10^3	1250	0.025%
Escherichia coli	1.6×10^8	0.05	0.000 80%

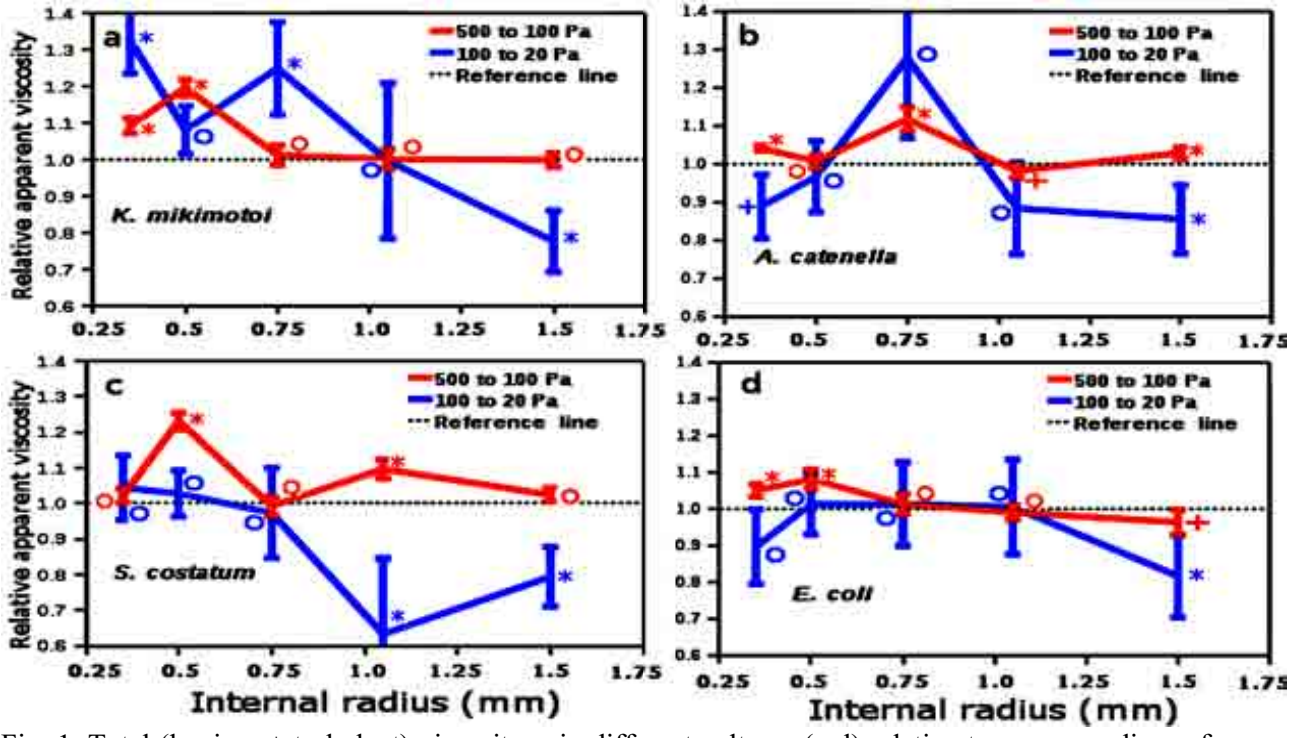

Fig. 1. Total (laminar + turbulent) viscosity η in different cultures (a-d) relative to corresponding reference water. It is shown for the high-*P* (500 to 100 Pa) and low-*P* (100 to 20 Pa) ranges of hydrostatic pressure difference. A relative viscosity of 1 indicates no difference from reference water. Error bars are SD. O – no sig. diff; + - sig. diff. $P < 0.1$; * - sig. diff. Prob. < 0.05. No. of replicates mostly 10 (7-11) for both experimental samples and reference water.

This happened at low Reynolds number (*Re* 340-68), (calculations in Jenkinson & Sun, 2014), which strongly suggests that the phenomenon is dominated by laminar drag reduction (LDR). Turbulent DR (TDR), that is caused by the elastic effects of polymers (Hoyt & Soli, 1965), may also have contributed to reducing RV at the higher values of *Re*, that is in the high-*P* range and at large *r*. The results are compatible with drag increase by rheological thickening caused by phytoplankton EPS, on which is superimposed LDR at low *Re* values. A contribution by TDR at the highest *Re* values (high *P,* high *r*) for *E. coli* (Fig. 1d) cannot be ruled out. What can explain the LDR that we found? In addition to the above mentioned laminar drag increase and TDR, both contributed by EPS, LDR has been modeled in suspensions of swimming flagellates (Thutupalli *et al*., 2011) and measured in coordinated-swimming (CS) in slowly sheared suspensions of *Chlamydomonas* and *E. coli* (Giomi *et al*. 2010) as well as *Bacillus* (Ishikawa & Pedley, 2007) at volume fractions φ of order 10% or even 3% (Gyrya *et al*. 2011). It seems unlikely, however, that CS could have caused the LDR in the present study as φ values were lower than 1% (Table 1) and, moreover, it occurred in culture of

S. costatum, which is non-motile. LDR in tube flow can also be caused by the Lotus-leaf effect, or superhydrophobic DR (SDR) (Rothstein, 2010). This is caused by fluid slipping over the inner surface of a tube bearing hydrophobic nm- to µm-sized irregularities. SDR tends to be greater when gas fills the spaces between these irregularities (Cassie state), and is weaker when they are occupied by the liquid (Wenzel state). Even if the capillaries become coated with EPS-derived hydrophobic irregularities, it is unlikely that they any zone of SDR they produced could occupy the bulk of a tube of r=1.5 mm, as maximum slip lengths achieved <200 µm (Lee *et al*, 2008). A more likely scenario would be that the surfaces of plankton and EPS particles suspended in the shear field would themselves be covered in hydrophobic irregularities, perhaps in the Cassie state due to O_2 production. Most algae remain very clean in nature, suggesting that their surfaces are self-cleaning and antifouling, as in many terrestrial plants and lichens (Shirtcliffe *et al*. 2006). Indeed the highest LDR we found was in *S. costatum*, known for its long fibrils (Castellví, 1969, Yamada & Takano, 1987), which may function as hydrophobic irregularities for producing SDR. Whatever the mechanism for producing the LDR that we measured, it is appears to be a phenomenon newly described for phyto- and bacterio-plankton, to which it gives another hitherto unsuspected tool to engineer (i.e. manage) their environment (Jenkinson & Wyatt, 1995; Wyatt & Ribera d'Alcalà, 2006) whether in HABs or in bioreactors.

These recent results show that phytoplankton at typical HAB concentrations, as well as bacterial culture, sometimes increase viscosity in laminar flow and sometimes decrease it. Nano- and micro-fluidics close to plankton surfaces have recently been reviewed (Jenkinson, 2014).

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HAB mitigation strategies in Korea and eco-friendly new initiatives

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Abstract

Due to the widespread and persistent fish-killing harmful algal blooms (HABs) during the last two decades in Korea, a variety of mitigation strategies have been introduced since 1996. These were designed to reduce the size of HAB populations and to abate their impacts. They include precautionary prevention and direct control of blooms. Monitoring and early warning are key issues for precautionary prevention to be taken just before the outbreak of HABs. The post-HAB mitigation actions, such as moving pens to shelter, airlift pumping and direct control by clay dispersal are on-going major emergent measures to minimize the impacts of HABs. Since 1996 flocculent clay has been widely dispersed on aqua-farms to control the fish-killing dinoflagellate, *Cochlodinium polykrikoides,* blooms. Acknowledging recent increasing frequency and severity of HABs, it is necessary to find eco-friendly mitigation strategies for practical use. Such strategies should secure the public safety, maintain ecosystem stability, have no residual effects and allow for wide application on fish farms. While conforming to the urgent need and ecological concerns, new initiatives such as highly integrated real-time HAB watch, early warning (HAB-WW) and eco-friendly HAB management and mitigation (HAB-MM) are essential projects for sustainable coastal aquaculture production. In addition, a bio-manipulation technology to replace the HAB biomass by harmless diatom blooms will be one of the HAB precautionary preventions (HAB-PP) stratigies.

Keywords: HAB impacts, precautionary prevention, early warning, mitigation, clay dispersal

Introduction

In Korea, much of public attention has focused on how to minimize the impacts of HABs due to the wide spread and persistent fish-killing dinoflagellate blooms, mainly by *Cochlodinium polykrikoides*, since 1995 (Kim 1997; Kim *et al.* 1999a). When finfish in cages were exposed to the blooms of *C. polykrikoides* exceeding 10,000 cells/ml, the fish died in a few hours. Aquaculturists have been concerned by the appearance of such uncommon mass mortalities of fisk-killing blooms. This has led both scientists and governments to find better contingency measures to reduce the impacts of HABs.

When it comes to HAB control in Korea, the first *in-vivo* control experiment using cupric sulfate and flocculant clays was done on *Prorocentrum triestinum* (Kim 1986). For field application, cupric sulfate was once dispersed in the Jinhae Bay, South Sea of Korea, in 1981 to control *Karenia mikimotoi* blooms. Clay has been dispersed in the South Sea since 1996 to control *C. polykrikoides* (Choi *et al*. 1998; Kim 1998). Both *in vivo* and *in situ* experiments had proved that dispersed clay can scavenge live dinoflagellate cells and lower the density of HABs below the fish-killing level. Since then, the clay has been dispersed by local governments in and around cages to protect cultured finfish from mass mortalitites since 2007. It is important to note that clay for field application should not include nutrients or toxic and harmful substances according to guidelines (NFRDI 1997). Besides the clay dispersal, an algalytic enzyme of α-mannosidase (Lee *et al.* 2000) and sophorolipid-yellow clay mixture (Lee *et al*. 2008) had been recommended as a feasible mitigative agent to control fish killing blooms. Contemporarily, indirect mitigative measures such as an alarm system, shield curtains (Kim *et al.* 1999b), and artificial upwelling in and around pen cages (Kim 2006) have been used to control fish killing HABs. However, most of those mitigative agents are not fully justified for field application in the context of safety and adaptability.

Such public concerns push HAB scientists to create eco-fiendly HAB control agents. However eco-friendly

precautionary prevention and direct control strategies are not yet available for the safe use in around fish farms.

While conforming to recent requests, Korea is initiating a new HAB research plan on the integrated real-time warning and the effective mitigation strategies for the upcoming decade. It will be focused on the integrated real-time HAB watch and warning system (HAB-WW) at the initiation stage, and eco-friendly HAB management and mitigation (HAB-MM) for the subsequent development of high abundance HABs. In addition, bio-manuplation research that induces diatom blooms to out compete HABs and clearing the bottom sediments of the benthic resting cysts to block recurrence of HABs will be promising components of the HAB-precautionary prevention projects (HAB-PP).

A review of available HAB mitigation strategies and the feasibility for field application

Until the late 1970s, no attention was paid to the direct control of HABs, because the prevailing species responsible for red tides were mostly diatoms (Kim 1997). In 1981 from July to September, persistent dense blooms caused by *Gymnodinium* type-65 (now *Karenia mikimotoi*) occurred in the Jinhae Bay, South Sea of Korea, and caused mass mortalities of cultured and wild fish and shellfish (Park 1982). At that time, cupric sulfate wrapped in a fine net was dragged by a ship around the pen cages to kill fish-killing dinoflagellates (Kim 1986; Rounsefell and Evans 1958). However, this was not recommended for use because dispersal of cupric sulfate was lethal to sensitive living organisms.

In 1995 widespread and persistent *C. polykrikoides* blooms accompanied by mass mortalities motivated the use of clay dispersal on fish farms. Since then, a variety of practical mitigation techniques (Kim et al. 1999b) have been employed directly or indirectly to minimize the harmful impacts and the size of HABs in Korea. Kim (2006) had classified them into two categories: precautionary impact prevention, and bloom controls (Table 1). Bloom control can be categorized as either direct or indirect, depending upon whether the effort targets an existing bloom or strives to reduce future blooms.

Up to now, a variety of biological, physical, and chemical direct control mechanisms have been used in attempts to directly control HABs. Of those, flocculant clays, top-down grazing, and algicidal agents were considered promising techniques (Table 2).

Table 1. Current mitigation and control strategies against HABs in Korea (Kim, 2006, modified)

Category	Before HAB	After HAB
Precautionary impact prevention	Monitoring Early warning Precautionary actions ○ early harvesting ○ less feed to fish ○ prepare mitigation facilities	Emergent actions ○ Move pens to refuge site ○ Water circulation ○ Oxygenation
Bloom controls	Indirect controls ○ Reduce nutrient inputs ○ Modification of water circulation ○ Bio-remediation	Direct controls ○ Physical control ○ Chemical control ○ Biological control

To date Korea (Kim 1987; Kim 1998), Japan (Shirota and Adachi 1976), China (Yu *et al*. 1994), and USA (Sengco *et al.* 2000) have dispersed clay to control fish-killing HABs. It is generally agreed that clay mitigation seems to be one of the promising techniques and has been recommended as one available for direct HABs control.

Table 2. The HAB controls and available agents (Kim 2006, modified)

Controls	Mechanisms	Available agents
Biological	Grazing	Copepod, ciliate, bivalves
	Algicidal	Bacteria, viruses
	Parasites	*Amoebophrya, Parviluciferai*
	Enzyme	Mannosidase, Sophorolipid
Physical	Destruction	Ultrasound
	Electrolysis	NaOCl
	Removal	Skimmer, screen filter
	Isolation	Shield curtain, perimeter skirt
Chemical	Flocculants	Clays and long-chain polymer
	Surfactants	Sophorolipid, aponin
	Mucolytic coagulants	Cysteine compounds
	Metals and liquids	Copper, $Mg(OH)_2$, H_2O_2

In the case of *C. polyykrikoides* blooms, clay showed high removal efficiency (Choi *et al*. 1998) and no significant impacts on the benthos (Kim, 2006). This allows local governments to purchase the clay and disperse it in and around the fish farm with the help of fishermen. The particle size of the clay selected for field use should be less than 50 μm, and dispersed at the concentration of 100-400

g/m^2. The clay minerals mixed with seawater are directed at the targed area at mid-day because the cells migrate to subsurface layers in mid-day (NFRDA 1997). Still in Korea, the long-term ecological risk assessment has been ongoing to clarify the potential impacts of the clays on the structure and function of coastal ecosystems.

When it comes to the physical controls, such as skimming, isolation, water bubble jets, ultrasonic destruction, centrifugal separation, and filteration, these are regarded as effective mechanisms to be available for high density and small scale blooms. In the case of a chemical control option, it shows little promise, because most of the chemicals are likely to be non-specific and thus will kill co-occurring algae and other organisms indiscriminatley. Especially in countries where raw fish is popular, like Korea and Japan, the use of chemicals will be more difficult due to the high sensitivity concerning the safety of raw fish.

In addition, several biological techniques such as algicidal bacteria, algalytic enzymes, and top-down grazing pressure have been recommended as possible mitigative techniques. These techniques can be used independently or with clay to enhance the removal rate of harmful algae. Among these, the algalytic enzyme (Lee *et al*. 2000) was once recommended as one potential control agent against *C. polykrikoides* because it demonstrated a wide spectrum of lytic activity toward the harmful algal species. With all its promise, α-mannosidase activity, was not practical due to its latent sluggsih lysis capability, the difficulty of mass production, and handling difficulties (Lee *et al*. 2000). As a means of top down grazing pressure, a large ciliate *Strombidinopsis* was also recommended as an efficient grazer (Kim *et al*.1999b). However, *Strombidinopsis* can not be applied owing to the difficulty of mass culture in a short time. Acknowledging the present situation, clay dispersal has been regarded as an efficient and low-cost HAB control agent with little harm to living organisms by Korean aquaculturists.

Eco-friendly new initiatives for HAB mitigation and precautionary prevention

As the algal blooms are becoming more and more harmful and widespread in part, due to eutrophication and climate change, we need to take new initiatives to minimize the impacts of fish mortalities and food poisonings from planktonic and benthic toxic micro-algae.

The first essential work before the outbreaks of HABs is to build an integrated real-time HAB watch and warning system (HAB-WW) to detect the initiation stage of HABs (Table 3). The HAB-WW in Korea will have two key components. The first will be the use of remotely sensed bio-chemical information acquired by satellite. The second will be the transmitted *in-situ* microscopic images capable of enumeration and identification of harmful algal species from cruises. The HAB-WW research project will start in 2014. In this effort, fibre optic microarrays (Anderson *et al*. 2006), molecular probe-based detection, and auto-identification microscopes equipped with software capable of species identification will be utilized similar to the HAB buoy (Culverhouse *et al*. 2006). In addition regionally-specific algorithms to identify blooms from satellite images are prime objectives to ensure practical application.

The new initiative of eco-friendly HAB-MM will be multidisciplinary with global cooperation. It is an important task to find new and innovative mitigation materials and/or techniques including the improvement of current mitigation strategies.

Table 3. New initiatives and strategies for HAB management and mitigation for the coming decade in Korea

Initiatives	Major tasks to be developed
HAB-WW	○ Microscope software capable of species identification and enumeration ○ Relevant algorithms capable of Identifying harmful species using satellite images ○ HAB- kit capable of toxin detection
HAB-MM	○ Highly efficient clay dispersal which may be combined with the other technologies ○ Dilute dense HABs to avoid mass-mortality by water circulation
HAB-PP	○ Replace HABs by harmless diatom blooms ○ Kill or remove resting cysts from the sediment ○ Utilize allelopathy to rearrange the prevailing harmful species

HAB-MM technique will require extensive testing for lethality, specificity, and general safety, and must surmount significant regulatory hurdles (Anderson *et al*. 2001) for field application on HABs. To improve on-going HAB-MM, enforcing

the efficiency of clay scavenging through modifying the structure of clays is highly recommended to reduce the amount of clay dispersed.

A method of bio-manipulation is to modify the structure of the ecosystem to conserve, establish, and re-establish a biological community that will prevent recurrences of HABs (SCOR/GEOHAB 1998). One promising project is the re-establishment of harmless diatom blooms to replace HABs through the man-made manipulation of sediment quality to increase the germination of diatom cysts. The aims of the clearance of benthic cysts of the HAB seed population is to suppress the consecutive outbreak of HABs (Ichikawa *et al*. 1993).

While acknowledging the inevitability of HAB control agents, all mitigation technologies should strive to be non-toxic, avoid bio-accumulation or residual build up on the benthos and preserve the stability of the marine ecosystem. Furthermore, it is obligatory to reduce the amount of clay dispersal to improve the efficiency and to lower the impacts on living organisms in the final field application during HAB events. The study of new initiatives and mitigation techniques for direct control of HABs should become an urgent subject to replace clay dispersal which is still controversial.

Conclusions

In the future, the magnitude of HABs will be increasing due to coastal eutrophication and geographical expansion owing to climate changes and ballast water transport (Hallegraeff 1993). Therefore, most countries have a willingness to pay great attention to HAB research focused on management and mitigation. Korea, one country suffering from HABs, is planning to build eco-friendly new initiatives of HAB-WW, HAB-MM, and HAB-PP as state-run project for the coming decade.The goal of this project is to secure public health and to protect aquaculture production. Based on the present available mitiation strategies such as the menthods to protect the fish cages from the areas affected by HABs, minimizing HAB effects, and directly controlling the targeted bloom populations in Korea, new initiatives and research projects will move forward with global cooperation and collaboration.

It takes a long time to develop eco-friendly initiatives which satisfy the safety requirements, are adaptable and consistent with the coastal environment, but it is must-do task of our generation.

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Introduction of harmful dinoflagellates through ship traffic: Differences between the Pacific and Atlantic coasts of Canada

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Abstract

The diversity and abundance of dinoflagellates in ballast water and ballast sediment was examined in >60 commercial ships from the Atlantic and Pacific coasts of Canada, focusing on potentially harmful species. Three categories of ships were compared: transoceanics (TOE), with mandatory ballast water exchange (BWE); coastal with BWE (CE); and coastal without BWE (CNE). Results show differences between the two coasts: mean concentrations were lower on the Pacific coast, where concentrations and number of taxa were greater in coastal exchanged ships and there was no significant difference between transoceanics and unexchanged coastal ships. On the Atlantic coast, transoceanics showed greater tank concentration and number of dinoflagellate taxa in ballast water and there was no significant difference between exchanged and unexchanged coastal ships. Ballast water exchange contributed little in our results to mitigate the risk associated with the transport of harmful dinoflagellates. The Atlantic coast of Canada thus receives more harmful dinoflagellates through shipping than the Pacific coast, and the discharge of ballast water introduces greater concentrations of harmful dinoflagellates than the discharge of ballast sediments. Our results also indicate that coastal ship traffic should be of greater concern, as it likely contributes to the spreading of harmful dinoflagellates.

Keywords: harmful dinoflagellates, ballast water and sediment, ballast water exchange, coastal Canada

Introduction

Ship traffic has increased steadily over the last decades and will continue to do so as international trade intensifies and because ship transport is considered less harmful for the environment than ground or air transport. To ensure ship stability, large volumes of water are generally pumped in ballast tanks and discharged when merchandise is loaded on board. Waters taken from one port in the world are thus discharged often far away from their original source. In Canada, Claudi and Ravishankar (2006) estimated that more than 52 million tonnes of ballast water are discharged annually in coastal environments. Shipping thus constitutes a potentially effective transport vector for aquatic organisms able to survive through the voyage. Dinoflagellates have characteristics that favour their survival in ballast tanks, notably mixotrophy and cyst formation. Several dinoflagellate species are harmful or toxic, and they have been implicated in ship-related invasions in some countries (e.g., Australia: Hallegraeff and Bolch 1991), sometimes with dramatic economic consequences. While foreign traffic is often thought to present the greatest risk, recent studies have pointed out that coastal shipping should not be neglected, notably because of secondary coastal spreading (Lawrence and Cordell 2010). Coastal eutrophication which often leads to increases in harmful algal blooms may also increase the risk associated with coastal shipping. To mitigate the risk of introduction of non-indigenous organisms, ballast water exchange was implemented in Canada in the early 1990's (now required by law: Canada Shipping Act 2006): ships traveling outside Canada's coastal waters must flush ballast tanks with open ocean seawater before being allowed to discharge ballast in Canadian waters. Since many harmful dinoflagellates have a coastal distribution, we hypothesized that ships that have undertaken BWE should show a low abundance of these species. This study, part of the Canadian Aquatic Invasive Species Research Network (CAISN), aimed to determine the abundance

and composition of the dinoflagellates present in ballast waters and ballast sediments of a large number of commercial ships visiting the East and West coasts of Canada, focusing on potentially harmful/toxic species, and to examine the influence of ship category (transoceanic or coastal) and ballast water exchange. Three categories of ships were examined: transoceanics (with mandatory BWE: TOE), coastal with (CE) and without BWE (CNE).

Material and methods

Ballast water was obtained from commercial ships visiting a number of ports on Canada's East and West coasts (>60 ships per coast: East coast: 22 TOE, 22 CE, 19 CNE; West coast: 28 TOE, 20 CE, 23 CNE). Most of these were bulk carriers, which dominate ship traffic in this region. Ships were selected on an opportunity basis, aiming for roughly the same number of ships in all three categories. Ballast water samples were taken with a 5 L Niskin bottle through manholes reaching the wing or top side tanks. Water was collected at four depths spread evenly from top to bottom of the water column, then mixed in 20 L Nalgene bottle. A volume of 13 to 19 L was sieved on-board through 73 µm, collected on 20 µm mesh then fixed with acid Lugol. Samples were kept at 4°C in the dark until microscopic examination within 12 months after sampling. Species were considered potentially harmful algae (HA) when listed in the IOC-UNESCO Taxonomic Reference List of Harmful Microalgae. Calcofluor was used when necessary to help identification. Cell toxicity was not determined. Only intact cells with clearly visible cell content were counted and assumed viable.

Ballast sediments were collected from approximately the same number of ships (East coast: 24 TOE, 21 CE, 20 CNE; West coast: 22 TOE, 22 CE, 20 CNE), although often not the same ships as for ballast water, since sampling required empty ballast tanks. Samples were taken from one ballast tank per ship, according to accessibility. Sediment samples were collected through direct access to the bottom of the tanks, after complying with security measures. Approximately 500 g of bottom sediments were collected from different areas within each tank, placed in a plastic bag and stored in the dark at 4°C. The total quantities of ballast sediments in a tank were estimated by measuring the surface area covered, the average thickness and the percent of coverage. In the laboratory, the sediment samples were mixed and a sub-sample of 5 cm^3 was diluted with filtered seawater and sonicated for 2-3 min, then sieved through 73 µm and 20 µm Nytex mesh. Dinocysts were identified and counted (>200 cysts per sample). Over 800 cysts were randomly selected and tested individually for germination in f/2 medium, as a verification of their viability (Casas-Monroy *et al*. 2011 and 2013).

Results

In ballast water, the most frequently observed potentially harmful dinoflagellate species were *Dinophysis norvegica*, *D. acuminata* and *Phalacroma rotundatum* for Canada's East coast, and *D. acuminata* and *D. norvegica* for the West coast (all associated with diarrhetic shellfish poisoning). Overall, there was at least one harmful dinoflagellate species in 81% of all ships examined on the East coast, but only 41% for the West coast. Average concentrations of harmful dinoflagellates in cells per litre were greater on the East coast than on the West coast (Fig. 1a). The greatest concentrations were found in CNE ships visiting the East coast, but the volume of ballast tanks in these ships was relatively small, hence concentrations expressed as cells per ballast tank showed reduced values compared to other ship categories (Fig. 1b). Results show that concentrations (per litre or per tank) and number of taxa were greater on the East coast, compared to the West coast. For the East coast, the greatest tank concentrations were observed in transoceanics (although per L values indicate lower concentrations in TOE ships), with no significant difference between exchanged and not exchanged coastal ships. For the West coast, the greatest concentrations were found in coastal exchanged ships, with no significant difference between transoceanics and not exchanged coastal ships. Hence, ships that undertook BWE did not show a clear reduction of the abundance of harmful dinoflagellates. In fact, the greatest tank concentrations and number of taxa (Fig. 1c) were observed in exchanged ships on both coasts.

For ballast sediments, the most frequently observed potentially harmful dinoflagellate cyst forms were *Alexandrium tamarense* (respectively in 32 and 19% of ships on the East and West coasts), *Lingulodinium machaerophorum* (= *L. polyedrum*) (in 28 and 27% of ships on the East and West coasts), *Polysphaeridium zoharyi* (= *Pyrodinium bahamense* var. *compressum*, Fig. 2) (in 32 and 8% of ships on the East and West

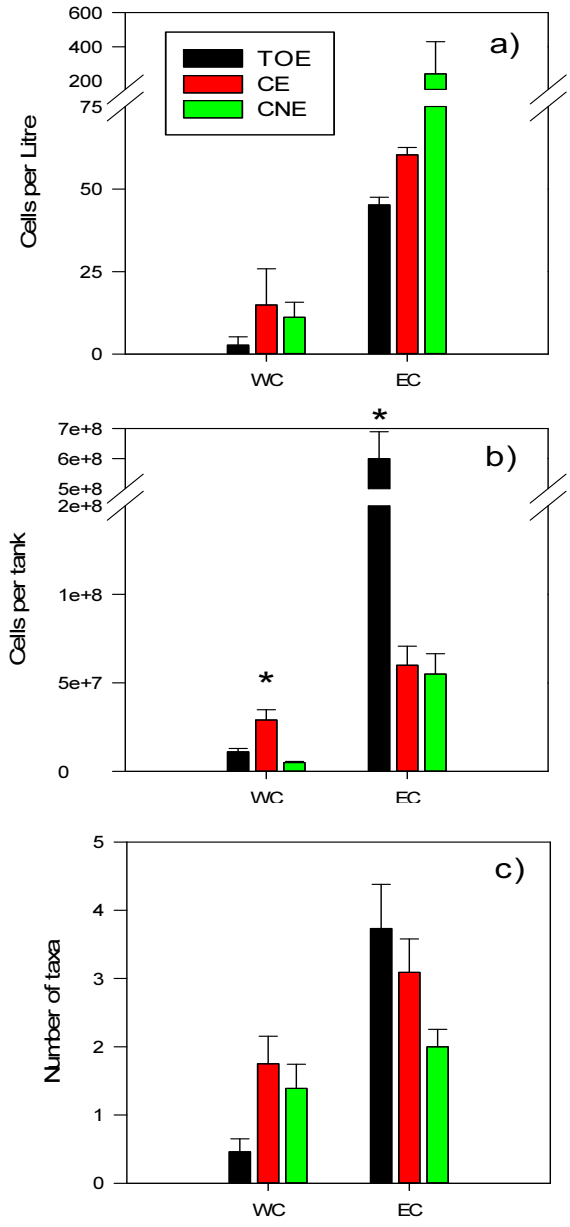

Fig. 1. Harmful dinoflagellate cells in ballast water from ships sampled on the West coast (WC) and East coast (EC) of Canada. Mean values with standard error for (a) cells L^{-1}, (b) cells per ballast tank, (c) number of taxa. *: significantly different from other ship categories (Permanova, $p<0.05$).

coasts) and *Gymnodinium catenatum* (in 17 and 10% of ships on the East and West coasts). Average concentrations per g of dry sediment showed greater values in CNE ships on the East coast and generally lower concentrations on the West coast (Fig.3a). When expressed per ballast tank (hence affected by the amount of sediment per tank), cyst concentrations also showed greater values on the East coast, with no significant difference associated with BWE on either coast (Fig. 3b). The number of taxa was greater on the East coast and was not influenced by BWE (Fig. 3c).

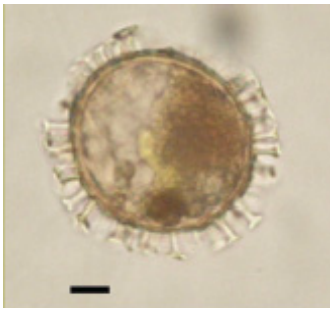

Fig. 2. *Polysphaeridium zoharyi* from ballast sediment

Discussion

Greater concentrations of harmful dinoflagellates (in ballast water and ballast sediments) were observed on the East coast of Canada, possibly related to the shorter duration of transoceanic trips in the Atlantic compared to the Pacific. Our results also show that undertaking BWE did not result in significantly lower tank concentrations of the target organisms, indicating the inefficiency of this measure when dealing with harmful dinoflagellates. A comparison with earlier studies on the East coast (prior to wide range implementation of BWE, e.g., Subba Rao *et al*. 1994) supports this, since it suggests little change in the presence and abundance in ships of several harmful dinoflagellates since BWE was enforced in Canada (Roy *et al*. 2012). Verification of the exchange is done by Transport Canada officers, by determining ballast water salinity before discharge. Comparison with geographic distribution maps (such as OBIS) suggests that some species (e.g., some *Dinophysis* sp.) may actually be picked up during BWE since they are present in the open ocean. A rough estimate of the number of harmful dinoflagellate cells discharged per year on each coast can be calculated based on cell concentrations and total ballast volume discharged per year for the East and West coasts of Canada (data courtesy of Dr. Sarah Bailey, Fisheries and Oceans Canada, Burlington). Values obtained (assuming all cells survive) range from $43 \cdot 10^9$ to $1198 \cdot 10^9$ cells discharged per year for the East coast, and from $0.3 \cdot 10^9$ to $8 \cdot 10^9$ on the West coast. A similar estimate was done for ballast sediment, assuming that 0.4% of bottom tank sediment volume was discharged

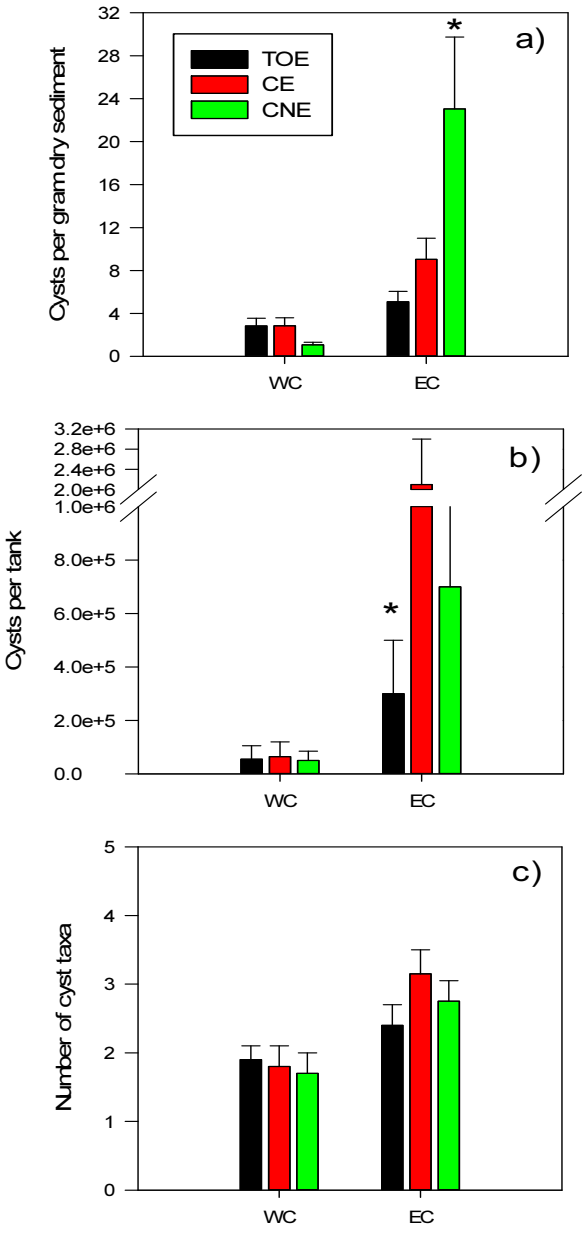

Fig. 3. Harmful dinoflagellate cysts in ballast sediments from ships sampled on the West coast (WC) and East coast (EC) of Canada. Mean values with standard error for (a) cysts g^{-1} dry sediment, (b) cysts per ballast tank, (c) number of cyst taxa. *: as for Fig. 1.

(Weise *et al.*, in preparation). Values were also greater for the East coast, but concentrations were less than for ballast water, ranging from $2 \cdot 10^6$ to $10 \cdot 10^6$ cysts discharged per year for the East coast, and from $0.02 \cdot 10^6$ to $2 \cdot 10^6$ for the West coast. Considering these estimates, ship traffic represents a risk for marine waters in terms of harmful dinoflagellates, particularly on Canada's East coast. Furthermore, coastal ships examined carried greater concentrations (in cells L^{-1} or cysts g^{-1}) than transoceanics. This may be indicative of secondary spreading from ports in the USA. On the West coast, ships considered "coastal" often originated from Asia with a stopover in the USA, resulting in roughly similar concentrations among the three ship categories, as observed for other categories of plankton (Villac and Kaczmarska 2011).

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Turbulence, Shear Stress, and Toxicity in *Heterosigma akashiwo*

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Abstract

Heterosigma akashiwo is a raphidophyte alga that forms ichthyotoxic harmful blooms. The toxicity of *H. akashiwo* varies from one bloom to another, within a single bloom, across time, and among locations. The possibility that shear stress induces toxicity was explored with this study. Cultures of *H. akashiwo* strain NWFSC-513 were grown in calm conditions, exposed to controlled shear forces with a Couette device, and then analyzed for toxicity. Shear treatments covered four intensities (0, 1, 4, and 10 s^{-1}) as well as three durations (20, 40, and 60 minutes). Two multiwell plate-based assays targeting haemolytic activity and cytotoxicity were utilized for measuring the putative characteristics of toxicity. The results revealed that toxicity scaled with culture density but shear intensity and duration did not appear to affect toxicity. Additionally, pipetting, stirring, shaking, vortexing, bubbling, and centrifugation were explored for effects on the *H. akashiwo* cells. A flow cytometer was used to measure algal density, resazurine dye was used to determine metabolic activity, and PAM fluorometry was used to measure photosystem efficiency. All procedures had measurable impacts on the indicators of cell survival except for pipetting, with the most dramatic impacts coming from centrifugation and bubbling.

Keywords: *Heterosigma akashiwo*, toxicity, turbulence, shear, physiology, HAB

Introduction

Heterosigma akashiwo (Hada) forms blooms which stain the water a distinctive red-hue and can be highly toxic to marine organisms (Hara and Chihara 1987). Large scale and devastating kills of both penned and natural finfish have been linked to the presence of *H. akashiwo* around the world (Smayda 1998). However, the mechanisms responsible for the toxicity of *H. akashiwo* remain elusive. Studies on raphidophytes have associated their toxicity with reactive oxygen species, red blood cell lysing haemolytic compounds, brevetoxin-like organic neurotoxin, and extracellular excretions of mucous and enzymes (Khan *et al*. 1997; Twiner and Trick 2000; de Boer *et al*. 2004; Twiner *et al*. 2004; Ling and Trick 2010).

The toxicity of *H. akashiwo* varies from one bloom to another, within a single bloom, across time, and among locations. Previous studies have linked changes in per-cell toxicity to environmental variables, but physical shear stress is one environmental variable that has not seen detailed exploration in literature for its potential role in toxicity (Haque and Onoue 2002; de Boer *et al*. 2004). The fragility of many toxic algae may have dissuaded researchers from pursuing shear stress since the combination inhibits growth (Juhl and Latz 2002; Basterretxea *et al*. 2005). This highlights the proposed paradoxical relationship between stress and toxicity, where increased stress may drive increased bloom toxicity but it may also prevent bloom formation. However, shear stress in marine environments is tied closely to wind, waves, and tides, which vary over time and location (Thomas and Gibson 1990).

It was predicted that altering the amount of shear stress after a growth-promoting calm period would overcome this paradox, yielding both biomass and the potential for stress-induced toxicity. It was hypothesized that a relationship would exist between shear forces and toxicity such that greater shear force would result in greater toxicity. Additionally, it was hypothesized that common laboratory techniques which function through physical forces would have measurable impacts on *H. akashiwo* cells.

Methods

H. akashiwo strain NWFSC-513 was used to inoculate cultures in autoclave sterilized, modified, ESAW

salt solution enriched with f/2 nutrients. 9 L Nalgene polycarbonate carboys filled with 5.4 L of medium were inoculated at 10% for a total culture volume of 6 L (Anderson *et al*. 2005). The cultures were maintained at constant temperature (18 °C) and a 12h:12h saturating light:low light irradiance cycle (200:10 µmol photons m^{-2} s^{-1}).

Cells from cultures at the early stationary stage of growth were exposed to shear stress controlled in a Couette device. This device created laminar shear proportional to the angular speed of the outer cylinder (Pasiac and Gavis 1975). Treatments included shear intensity of 0, 1, 4, or 15 reciprocal seconds for durations of 20, 40, or 60 minutes. The cells or cell products were then concentration-standardized to 200, 100, or 50 cells / µL by centrifugation (2000 x g; 5 minutes) and subsequent re-suspension in modified volumes of supernatant before being lysed through two cycles of freeze-thaw. The lysed samples were assessed for changes corresponding to toxicity.

Standardized algal samples were used to test for both haemolytic activity and cytotoxicity as putative characteristics of toxicity. Hemolytic activity was measured in an erythrocyte lysis assay similar to those described by Eschbach *et al*. (2001) and Ling and Trick (2010). Rabbit erythrocytes were used as described by Ling and Trick (2010) but following a higher-throughput protocol using micro-well plates as described by Eschbach *et al*. (2001). Cytotoxicity was determined in a rainbow trout (*Oncorhynchus mykiss*) gill cell-line based cytotoxicity assay similar to those described by Dayeh *et al*. (2003) and Dorantes-Aranda *et al*. (2011). Trans-well membrane inserts were not used and the Alamar Blue protocol was modified to use Presto Blue (Life Technologies Inc., Burlington, ON, Canada). Additionally, cultures were sampled during exponential growth and exposed to common laboratory procedures (Table 1). A flow cytometer was used to determine culture density, Presto Blue resazurin-based dye was used to approximate metabolic activity, and PAM fluorometry was used to determine photosystem efficiency as F_v / F_m for dark-adapted samples.

Metabolic activity was standardized by subtraction of the mean blank fluorescence prior to normalization. ANOVAs, t-tests, and graphs were produced using R (R Core Team 2012).

Results

Cell density impacted toxicity such that greater *H. akashiwo* density caused greater loss of viability for both the haemolytic assay erythrocytes as well as the cytotoxic assay RTgill-W1 cells [Fig. 1;

Table 1. Common laboratory procedures that apply physical forces to samples, defined for reproducibility.

Procedure	Device	Intensity	Duration	Notes
Control	Eppendorf Xplorer electronic pipette (Wide Orifice Tip)	Speed setting 1	1 x	
Pipetting	Eppendorf Xplorer electronic pipette (Standard Tip)	Speed setting 7	100 x	
Vortexing	VWR Mini Vortex MV1	2500 rotations / min	3 x 5 sec	Samples canted between repetitions
Shaking	Quick hand motions, flicking at the wrist	~1 motion / sec (Subjective)	15 sec	
Stirring	VWR Lab Disc Magnetic Stirrer Octagon Stir Bar	60 rotations / min	30 min	
Bubbling	Aquarium air pump Ceramic air stone	~5 L / min	30 min	0.2 µm filtered air
Centrifugation	Beckman Coulter Avanti J-E with JS-5.3 rotor BD Falcon 50 mL conical tip centrifuge tube	2000 x g; 15°C	5 min	Resuspended by pipetting with same technique for control (modified with 5 repetitions)

Haemolytic: $F(2,285) = 128$, $p < 0.01$; Cytotoxic: $F(2,285) = 109.8$, $p < 0.01$)]. Neither greater shear intensity or longer shear duration were shown to impact toxicity (Haemolytic: Intensity: $F(3,284) = 1.078$, $p = 0.36$; Duration: $F(2,285) = 0.432$, $p = 0.65$; Cytotoxic: Intensity: $F(3,284) = 1.929$, $p = 0.12$; Duration: $F(2,285) = 1.265$, $p = 0.28$).

Stirring, centrifugation, and bubbling were each seen to reduce algal density [Stirring: $t(12.4) = 5.32$, $p < 0.01$; Centrifugation: $t(8.5) = 8.88$, $p < 0.01$; Bubbling: $t(14.0) = 19.9$, $p < 0.01$]. Conversely, algal density was not impacted by pipetting, vortexing, or shaking ($p > 0.05$ for each).

Metabolic activity increased after centrifugation and shaking but decreased after stirring and bubbling [Centrifugation: $t(38.8) = 6.11$, $p < 0.01$; Shaking: $t(61.9) = 6.24$, $p < 0.01$; Stirring: $t(49.6) = 7.07$, $p < 0.01$; Bubbling: $t(55.8) = 8.43$, $p < 0.01$]. It was not impacted by pipetting or vortexing ($p > 0.05$ for both).

Photosystem efficiency, as measured by F_v / F_m, was reduced by bubbling, vortexing, and centrifugation [Bubbling: $t(54.2) = 2.67$, $p = 0.01$; Vortexing: $t(60.5) = 4.81$, $p < 0.01$; Centrifugation: $t(59.8) = 6.68$, $p < 0.01$]. It was not impacted by pipetting, stirring, or shaking ($p > 0.05$ for each).

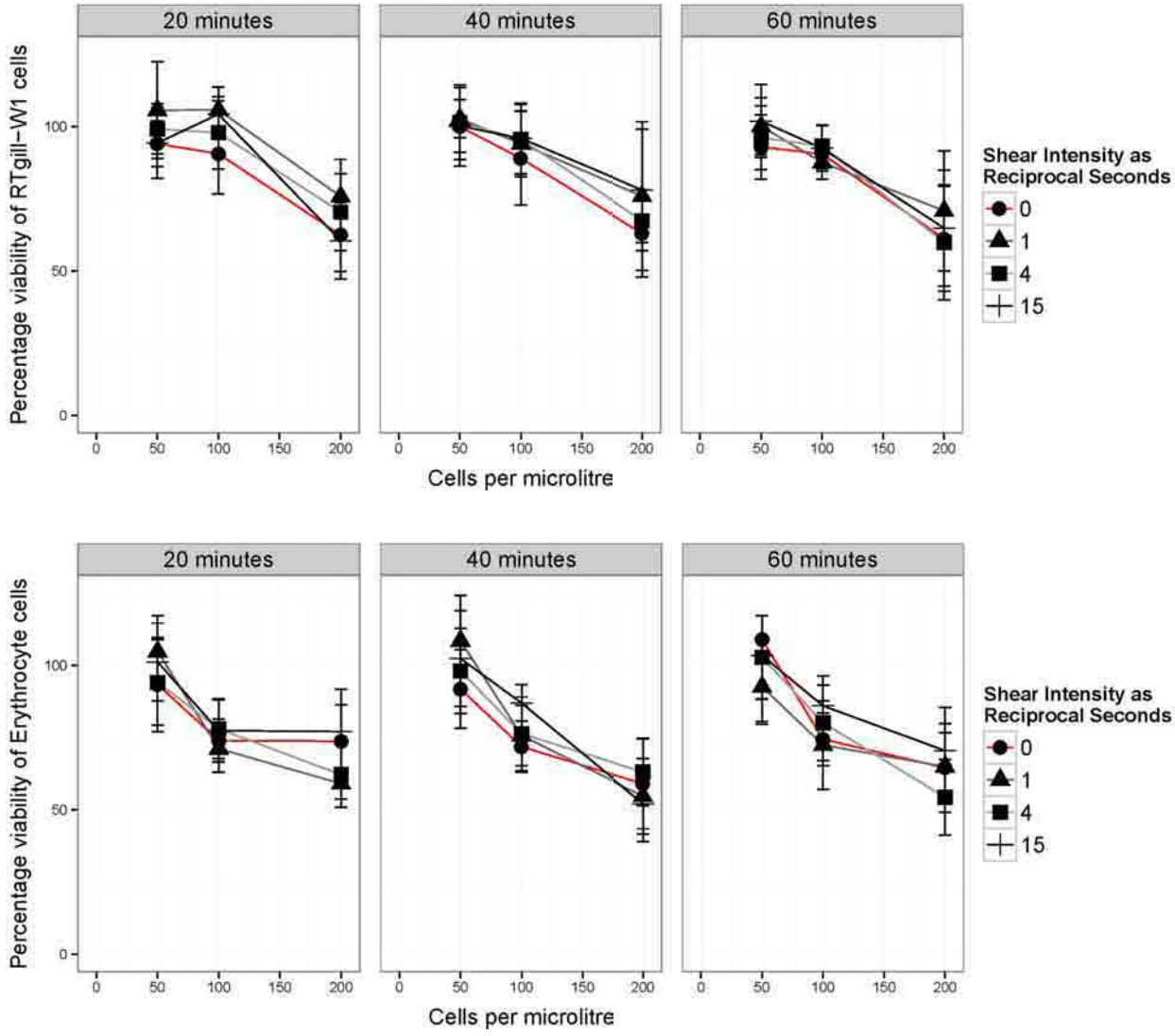

Figure 1. The viability of rabbit erythrocytes and rainbow trout gill cells after 6 and 24 hours of exposure, respectively, to shear-treated and freeze-thaw-lysed Heterosigma akashiwo samples. Shear treatments included four intensities and three durations. Exposure treatments included three culture densities. Error bars represent 95% confidence intervals.

Discussion

The observed *H. akashiwo* density-dependent toxicity responses were in agreement with the results of previous studies (Ling and Trick 2010, Dorantes-Aranda *et al*. 2011). Nonetheless, the hypothesis that toxicity would be affected by shear stress was not supported by the findings. Neither shear intensity or duration correlated with significant patterns of change in the viability of either of the erythrocytes or RTgill-W1 cells (Fig. 1). However, it is possible that the protocols employed in this study were not sensitive to changes in some toxicity factors. For example, the freeze-thaw treatment of the algal samples could deteriorate sensitive biochemical products that could otherwise have been involved in the toxic mechanism.

Experiments with common laboratory procedures revealed that these procedures are not benign (Table 1). The most extreme effects were arguably the reduction in counted cells after stirring, centrifugation, or bubbling. These reductions indicated ruptured or removed cells and were confirmed with microscopy.

Reduced metabolic activity observed after stirring and bubbling were supported by the cell losses but the increased metabolic activity observed after shaking and centrifugation were not expected. Since metabolism was measured indirectly though reduction of resazurin dye, it is possible that the increase was due to unexplained chemical mechanisms other than metabolic reduction.

The losses of photosystem efficiency were especially interesting since they implied that the cells which were not lost completely still suffered impairment by the procedures.

These findings should be considered in the context of the considerable interstrain variability documented for *H. akashiwo* (Fredrickson *et al.* 2011). An examination using other strains is required to evaluate species-level variations.

Conclusions

The results of this study suggest that shear forces are more likely to affect bloom toxicity through control of biomass than through per-cell toxicity. This study also revealed that routine laboratory procedures can be confounding factors and must be continually reassessed for their suitability. This will be especially true when novel questions are posed for exploring the toxic mechanisms of *H. akashiwo* and other fish-killing algae.

Acknowledgements

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Exploration of Phoslock® clay in mitigating *Prymnesium parvum* fish-killing algal blooms in aquaculture ponds

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Abstract

When in 2009 a tropical Australian barramundi farm suffered from fish-killing *Prymnesium parvum* blooms, the farm manager decided to manipulate N:P nutrients by adding Phoslock® bentonite clay and adjusting pH through the addition of molasses. Phosphate levels were maintained at <0.03 mg/L, phytoplankton biomass and dissolved oxygen became more stable and *Prymnesium* disappeared from the system altogether. We conducted *Prymnesium* culture experiments under different N:P ratios to interpret field observations, while measuring toxins using the RTgill-W1 cell line assay. Methanol extracts of nitrogen deficient cells were the most toxic during stationary phase, followed by phosphorus deficient cells and with nutrient sufficient cultures least toxic (7, 20, 65% gill cell viability, respectively). Sonicated whole-cell cultures were more toxic after 4h (0-13% gill cell viability) but lost significant toxicity after 24h in the dark, suggesting toxin degradation. Phoslock® effectively removed 60-100% of water soluble toxicity of live *Prymnesium* and offers great potential as on-farm emergency response. We are currently refining clay type and dosage, including exploration of application of clays to other ichthyotoxic algae.

Keywords: *Prymnesium parvum*, fish killers, Australian barramundi farm, Phoslock® clay

Introduction

Pond-based barramundi (*Lates calcarifer*) aquaculture is currently practiced by three Darwin companies and valued at $4.3M per annum. These shallow (1.5m) large ponds (2.5-3.25 ha; 24-31°C, 10-38 salinity) are eutrophic (receiving 150kg feed/ha/day) and ecologically unstable. In May/June 2009 one farm suffered significant loss (Aus $95,000) of fish linked to a bloom identified by light and transmission electron microscopy as the haptophyte *Prymnesium parvum*, which was successfully established in culture (PPDW02 strain). *Prymnesium* algal blooms have became a routine problem for the Israel Tilapia (since 1947) and Texas striped bass pond-based aquaculture industry (since 1985; Roelke *et al.* 2011). Blooms vary considerably in toxicity, from being virtually non-toxic under balanced nutrient conditions (N:P=16:1) to generating fast, irreversible, fish gill damage under conditions of N and P deficiency (Johansson & Graneli 1999). This offers the potential to manage and mitigate these fish killing algal blooms by manipulating nutrients in aquaculture ponds (Kurten *et al.* 2007), by the addition of ammonium sulphate (practised in Israel) or manipulating phosphorus e.g. using Phoslock® clay (Ross *et al.* 2008). This bentonite clay is applied as a slurry in which reactive lanthanum ions irreversibly bind phosphate ions, but its effectiveness is strongly influenced by pH. Confronted with a farm threatening *Prymnesium* bloom in 2010 the manager engaged in a pond manipulation experiment adding Phoslock® (30 kg /kg phosphate) and adjusting pH<7.7 by the addition of molasses which also stimulates microbial communities (Body 2011; Fig.1). Phytoplankton biomass became more stable, with reduced diurnal oxygen fluctuations (compare Fig.2, top and bottom), and *Prymnesium* disappeared from the system. By contrast, an untreated control pond developed a further *Prymnesium* bloom in June 2011 and again in March 2012 which killed Aus $10,000 worth of mullet. We here conducted *Prymnesium* culture experiments under different N:P ratios to interpret field observations, while measuring toxin production using the RTgill-W1 cell line assay. Phoslock® was applied to both sonicated and live algal cultures to assess effectiveness in removing *P. parvum* cells and their toxins.

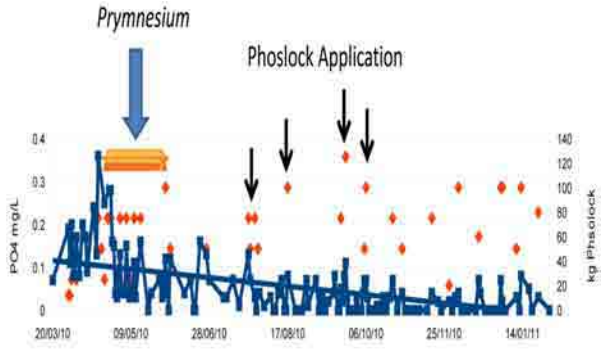

Fig.1. Application of Phoslock (diamonds and arrows) to a barramundi aquaculture pond reduced phosphate and removed blooms by *Prymnesium*.

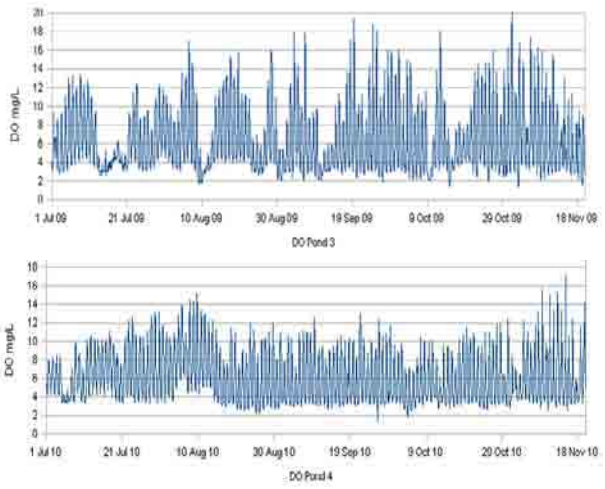

Fig.2. Diurnal fluctuations in dissolved oxygen levels in a control pond (above) and Phoslock-treated pond (below).

Material and Methods

Prymnesium parvum was grown in artificial sea water (35 salinity, pH 7.5) with N:P ratios adjusted to 16:1, 80:1 and 8:1. Cultures were grown in 3 L medium and maintained at 19±1°C at 120 µmol photons.m^{-2}.s^{-1} of light (12:12 L:D). 12d old cultures were diluted in artificial seawater to 10^5 cells.ml^{-1} and 400ml subsamples randomly assigned to the Phoslock® (1.54 g.l^{-1}) and control treatments. Toxicity of all samples was assayed with the RTgill-W1 cell line assay, using Transwell® permeable supports as per Dorantes-Aranda *et al.* (2011). As an indicator of gill cell viability, cellular metabolic reduction of the dye Resazurin to fluorescent Resorufin was quantified in a plate reader (BMG Labtech) and here reported as percentage viability compared to a non-toxic control (seawater).

Results

The gill cell line assay (toxin detection level 10^2 algal cells.ml^{-1}) proved orders of magnitude more sensitive than a comparative horse blood erythrocyte lysis assay ($6.2.10^5$ cells.ml^{-1}) in detecting ichthyotoxicity (results not shown). MeOH extracts of N deficient cells were the most toxic during stationary phase (93% gill cell mortality), followed by P deficient cells (80%) and nutrient sufficient cultures least toxic (35%). The gill assay is not sensitive to salinity, can be readily used with living *Prymnesium* cultures, and thus was used for all further work.

Application of Phoslock® to *P. parvum* cultures in the dark significantly increased viability of gill cells (Fig. 3 top) while Phoslock® itself had no impact. N limited initial cultures again proved most toxic, followed by balanced and P-limited cultures (24, 32, 53% gill cell viability, respectively). While toxicity under NP balanced conditions could be completely removed 4h after addition of Phoslock®, significant gill cell damage was still observed in P and N limited treatments (100, 75, 60% final gill cell viability, respectively). This gain in gill cell viability from Phoslock® addition was largely lost however after 3d incubation in the dark (Fig. 3 bottom).

Freshly sonicated cultures (4h) were more toxic than live cells (0, 0, 13% gill cell viability in –N,-P and NP treatments; Fig. 4, top) and again the greatest improvement in gill cell viability following Phoslock® addition was observed in the NP balanced treatment (improved up to 80% gill cell viability). Toxic potency of sonicated cells was very significantly reduced however after 24 h in the dark (Fig. 4, bottom). Phoslock® itself did not negatively influence gill cell viability (Fig.4. Phoslock® controls).

Discussion

The *P. parvum* toxic principle remains incompletely known. Early work claimed cytotoxic, hemotoxic and ichthyotoxic activity towards a range of organisms (Shilo 1967). Igarashi *et al.* (1999) characterised prymnesin 1 and 2, with the latter claimed to be the major hemolysin. Henrikson *et al.* (2011) however were unable to detect prymnesins in field and lab samples and proposed undescribed polyunsaturated fatty acids as ichthyotoxins. Methanol has been widely used to prepare *Prymnesium* extracts for erythrocyte lytic assays (Eschbach *et al.,* 2001), but the production of extracellular compounds

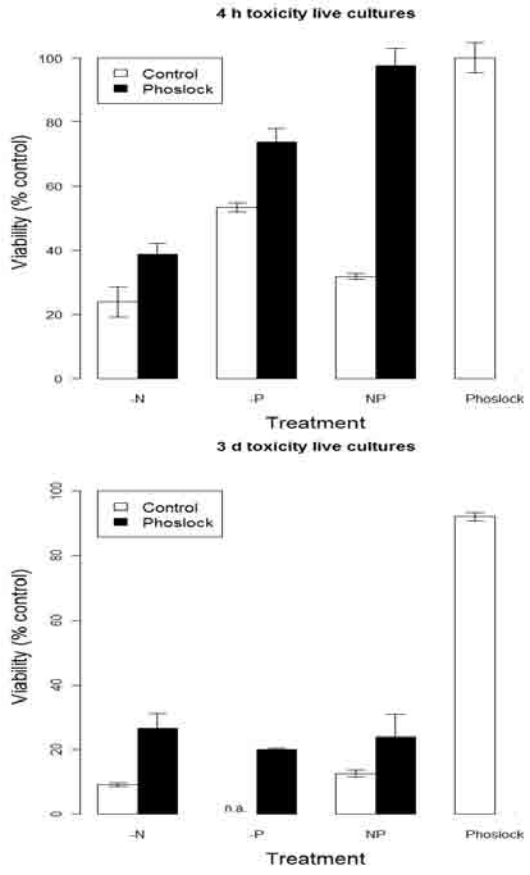

Fig.3 Toxicity of live algae after 4 h (top) and 3 d (bottom) exposures of gill cell lines to control and Phoslock® treated cultures. Black and white columns represent treatment (-N, -P, NP) and control (no Phoslock). N.a.: not available.

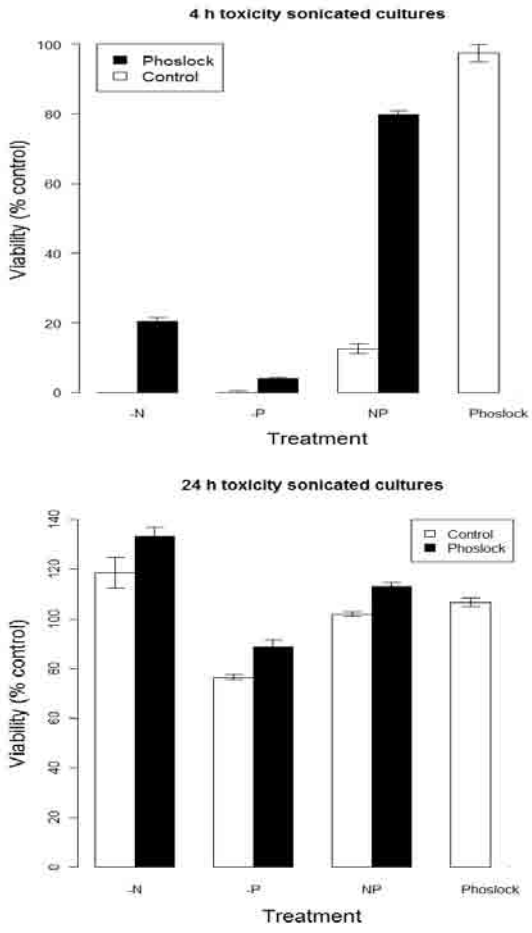

Fig.4. Toxicity of sonicated *P. parvum* 4 h and 24 h after Phoslock® application.

(Fistarol *et al.,* 2003) suggests that the alga produces both lipophilic and water soluble toxins.

In the present work, the application of the sensitive RTgill-W1 cell line assay suggests the continuous excretion of water soluble toxins by live algal cells (Fig. 3, compare 4h and 3d exposures). While sonicated cells temporarily released large amounts of toxins, these subsequently rapidly degraded even in the dark (Fig. 4, compare 4h and 24h exposures).

Clay flocculation has been widely explored as a mitigation strategy to remove algal cells from the water with removal efficiency dependent on type of clay (Sengco *et al*., 2005). In the present work the phosphate adsorbing Phoslock® was successfully used in mitigating *Prymnesium* blooms in an aquaculture pond, but a much greater benefit appeared the removal of 60-90% toxicity. Such property of clay has previously been documented for *Prymnesium* by Sengco *et al* (2005), but also applies to brevetoxins (Pierce *et al*. 2004) and microcystins (Prochazka *et al.* 2013; summarised in Table 1). We are currently refining clay type and dosage, including exploration of clays to try and mop up ichthyotoxins from other

Table 1. Removal of exudte toxins by clay

		Removal	
Prymnesium (gill toxicity)	Phoslock (95% bentonite + 5% lanthanum; particle size in slurry: 95% <11 µm, peak at 3 µm; 1.5g/L)	60-90% (N:P dependent)	Present work
Prymnesium (haemolysis)	Wet bentonite SWE4 (0.5-5g/L)	64-77%	Sengco et al. 2005.
Extracellular Brevetoxins	Phosphatic Clay IMC-P2(0.25g/L)	90%	Pierce *et al.* 2004.
Microcystins MC-LR	Bentonite (5-50g/L)	90	Prochazka *et al.* 2013.
Microcystins	Korean Yellow Clay (5-50g/L)	60	Prochazka *et al.* 2013.

algae such as *Cochlodinium, Heterosigma, Chattonella and Karlodinium*.

Acknowledgements

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Beyond the Bloom: Using a Socio-Ecological Systems Framework to Investigate Stakeholder Response to Harmful Algal Bloom Management in the Chesapeake Bay, USA

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Abstract

Summer blooms of toxic Microcystis aeruginosa have become increasingly problematic in tributaries of the Chesapeake Bay, and lakes and ponds of the watershed, spurring concerns over associated health risks, environmental degradation, and prompting various management and mitigation efforts. Effectively abating M. aeruginosa, however, requires investing in human dimensions research that looks beyond the immediate bloom site to understand how broader socio-ecological systems influence stakeholder response to harmful algal blooms and harmful algae management. This paper presents a comparison of two sites that apply different management approaches to control M. aeruginosa to demonstrate how considering broader socio-ecological system dynamics aid in developing resilient management approaches for impacted communities.

Keywords: socio-ecological systems, resilience, Chesapeake Bay, *Microcystis aeruginosa*, nutrient management, mitigation

Introduction

The Chesapeake Bay is the largest estuary in the US, with a watershed that extends across 64,000 square miles, six states and Washington, DC. This wide, shallow, brackish estuarine system provides ideal habitat for culturally and ecologically important blue crab, oyster, and striped bass fisheries. Regional population growth and expanding agriculture throughout the watershed, however, are affecting the health of the Bay through increased nutrient loads (Fisher *et al*. 2010; Kemp *et al.* 2005). One output of high nutrients is increasingly persistent *Microcystis aeruginosa* blooms in many fresh and tidal-fresh water tributaries, lakes and ponds (Tango & Boynton 2008; Fisher *et al.* 2006). These toxic blooms create health concerns, and impact local tourism and industries as they inhibit water usage, and increase regulatory pressures on agriculture and other industries.

Regional efforts to control *M. aeruginosa* align with predominant harmful algal bloom (HAB) management practices used in the US (Lewett *et al*. 2008). Advisories are posted following blooms to alert vulnerable populations of potential health risks. Monitoring programs have been established by the National Oceanic and Atmospheric Administration and the states of Maryland, Virginia, and Delaware to track bloom occurrence and predict seasonal trends for HAB. Extensive investment in nutrient management is also occurring across the Chesapeake Bay watershed through a series of regulatory frameworks, established by the 1983 Chesapeake Bay Agreement to coordinate regional restoration goals. Increasingly frequent blooms of *M. aeruginosa* are also prompting interest in developing alternative mitigation techniques, such as clay flocculation and barley straw mitigation (Sellner *et al.* 2012).

This paper applies a socio-ecological systems (SES) framework to investigate how stakeholders respond to HAB management, providing insights to enhance future management strategies. A SES approach rejects traditional dichotomies between human and natural systems to establish that they are intricately intertwined in ways that dynamically affect social and biophysical structure and function across time and space. Alteration to any one component of a SES can trigger a range of responses across a system that

may result in potential positive, negative, or neutral change to overall system dynamics (Gunderson and Holling 2002, Adger 2000). By adapting the holistic perspective of SES, HAB management can more easily identify the primary social and ecological factors that prompt HAB formation, anticipate such events, and more effectively respond to HAB when they occur (Walker and Salt 2006). This requires not only understanding the ways that humans contribute to eutrophication processes, but also identifying underlying socio-cultural, socio-political, and socio-economic dynamics that inhibit and/or promote positive behavior change. Management that fails to consider the social implications of HAB gain only a partial understanding of bloom dynamics, and lack insights needed to affect community response. Their shortcomings may ultimately enhance long-term vulnerabilities of both human communities and ecosystems (Berkes *et al*. 2006). Using two case studies, this paper demonstrates how HAB managed through a SES framework builds adaptive capacity and enhances system resilience by enabling environmental intervention that meets both ecological and community needs.

Methods

Mattawoman Creek and Williston Lake in the state of Maryland were selected for this study, as both have experienced reoccurring blooms of *M. aeruginosa* and provide sites with contrasting management approaches: one developed by an external management group for local community implementation; the other a community-driven management plan. At Mattawoman Creek, consistent blooms during the summers of 2004, 2005, 2006, and 2008 ("Eyes on the Bay" 2012) prompted state managers to develop an ecosystem-based management plan (EBMP) to support local smart-growth initiatives. The community rejected the EBMP recommendations, however, because they failed to meet community needs. In contrast, severe *M. aeruginosa* blooms on Williston Lake during the summers of 2010-11 led to successful implementation of community-initiated management strategies. Both the successes and failures of these case studies provide important insights on how to effectively engage communities in HAB management.

Data were collected using an ethnographic approach. At Mattawoman Creek, this entailed using blogs, public video from county commissioner meetings and public hearings, newspaper articles, as well as online commentary on the EBMP and county smart-growth plans. These outlets were analyzed for stakeholder attitudes towards the EBMP. Participant observation was conducted at EBMP development meetings, and content of the final EBMP report was analyzed for inclusion of local socio-economic, cultural, and political considerations to assess how objectives and recommendations align with community needs and concerns. At Williston, participant observation at community meetings was completed, as well as hour-long semi-structured interviews with 27 key-informants. Questions were open-ended and used to elicit knowledge about M. aeruginosa and other HAB, perceived challenges, opportunities, and risks, as well as preferred solutions. Each interview was transcribed and analyzed using text analysis software (ATLAS.ti 2010®).

Mattawoman Creek: Managing HAB without a SES Approach

The Mattawoman Creek is often considered an exemplary Chesapeake small watershed because it has remained relatively pristine due to slow growth. It is located within a prosperous county that prides itself on its small-town communities and agricultural roots. Mattawoman Creek has become a popular destination for recreation, prompting local government interest in ecotourism. The bass fishery alone brings in $40 million annually (Conn *et al*. 2011). Encroaching urbanization from Washington, DC, however, is threatening the health of Mattawoman Creek. *M. aeruginosa* blooms, among other signs of degradation, prompted state government to develop an EBMP to provide recommendations for county government to implement in future smart-growth initiatives (2011). The recommendations aim to protect ecological resilience by promoting high-density redevelopment outside the watershed, and placing limits on new development within the watershed. By doing so, Mattawoman Creek will be protected from excess nutrients that accompanies impervious surface expansion and deforestation, and promotes HAB. The authors of the report employ HAB as a water quality indicator and measure of fishery health. There is little discussion of human health or socio-economic impacts of HAB in Mattawoman Creek, nor any in-depth assessment of stakeholder needs or drivers, suggesting that the report authors strictly adhere to an ecological framework in their assessment. This aligns with an

emergent theme in EBMP development discussions encapsulating the belief that ecologically-directed management that protects resources from human encroachment will indirectly benefit local communities by creating a healthier and safer environment in the long-term. Limiting the scope of the EBMP to only survey ecological factors of the Mattawoman Creek SES, however, creates social vulnerabilities, as community needs are not addressed. Communities that sense increased vulnerability will often choose alternative pathways (Smit and Wandel 2006), which is precisely what happened at Mattawoman Creek.

Ultimately county government rejected the EBMP report because of misaligned values and beliefs about risk (Rothstein *et al.* 2006). For the authors of the EBMP, Mattawoman Creek is at risk of reaching irreversible thresholds that would prevent the system from returning to a sustainably healthy state. By restricting development in the Mattawoman Creek Watershed, it seeks to protect ecological vitality, and through this promote long-term community health. For county officials, however, community health is at risk without the economic support of the Creek. They argue that it is possible to promote smart-growth development that does not disconnect local communities from the Mattawoman Creek, maintaining that close community ties to the Creek is what promotes local environmental values to protect it. The report authors have missed an opportunity to discuss water quality degradation in ways that are tangible for stakeholders. Harmful algal blooms can be used as such a tool, as will be demonstrated with Williston Lake.

Williston Lake: Value of a SES Approach

Williston Lake is a manmade lake located in a rural, agriculturally dominant county. It is privately owned by the Girl Scout Council of the Chesapeake Bay, a non-profit organization that provides activity-based mentorship programs to children. The Lake is used recreationally by a local Girl Scout camp and lakefront landowners. Decadal nutrient contributions from the surrounding landscape have slowly eutrophied Williston Lake, resulting in severe *M. aeruginosa* blooms during the summers of 2010-11 that restricted recreational use.

Several key actors facilitated response to the Williston Lake blooms. These include a 2010 nutrient reduction mandate known as the Chesapeake Bay Total Maximum Daily Load, which is driving Federal, state, and local government efforts to curtail nutrient inputs to the Chesapeake Bay. As a result, government agencies not otherwise involved in mitigation took interest in Williston Lake as an opportunity to meet nutrient reduction goals. The camp manager also played a significant role as a nexus between stakeholders involved in Lake remediation. Scientists were instrumental in providing access to data, instruments, and knowledge about *M. aeruginosa* and HAB controls that helped shaped community response. Analysis of ethnographic data reveal that the Williston Lake HAB triggered new social networks to form across local, state, and regional scales that facilitated relationship development between disparate stakeholders. Each stakeholder group took interest in Lake remediation for diverse, and sometimes conflicting reasons attributed to how they conceptualize the Williston Lake system. The Girl Scouts and landowners, for example, interpret *M. aeruginosa* health risks differently from scientists and heath officials. One local resident likened the blooms to living next to a nuclear power plant, while a health official compared the risks to a poison ivy rash. These responses are largely directed by previous knowledge frames of HAB, relationships to Williston Lake, values, and experiences -- factors that may also explain conflicting views about which mitigation solution is best for Williston Lake. The local community's recreational values prompted these stakeholders to seek solutions to immediately restore water access, despite possible socio-economic implications of routine application. Environmental managers, however, viewed these short-term, temporary investments as wasteful, pushing instead to implement long-term nutrient controls that would address the root of the problem while also contributing towards their broader nutrient reduction goals.

As relationships between divergent stakeholders developed, new knowledge formed and values merged, providing a platform to develop resilient solutions for Williston Lake that maintain a healthier lake ecosystem though local community support. While some conflicting viewpoints were maintained, stakeholders agreed that HAB mitigation must be affordable, scientifically supported, and eliminate associated health risks. Management representatives and the local community also became invested in stakeholder partnerships as a mechanism to maintain a robust decision-making process that supports multiple needs while also ensuring scientifically sound implementation. In the end, stakeholders agreed to invest in short- and long-term controls that

met local community desires for lake access, and assisted environmental managers in meeting nutrient reduction goals. These solutions included annual drainage to flush the Lake, and routine barley straw mitigation. The Lake is currently being considered as a demonstration site for county nutrient management plans. Williston Lake demonstrates how considering social and ecological dynamics in decision-making processes enhances resilience. Through partnership with scientific and management communities, the Girl Scouts and lakefront residents are now armed with knowledge about M. aeruginosa and nutrients that allows them to appropriately respond to HAB. Additionally, county, state, and Federal-level management bodies are able to more easily meet regulatory goals through access to strong social networks with the local community. By understanding the factors affecting stakeholder drivers and concerns, HAB can be managed in ways that sustain community needs while also enhancing the long-term health of the Williston Lake ecosystem.

Conclusion

Harmful algal blooms provide a tangible link to connect communities to abstract ecological processes (e.g., eutrophication), and the Williston Lake case study demonstrates how HAB can be used to engage communities. Harmful algal blooms also provide a tool to understand how social dynamics interact with ecological processes to affect system response. Investigating the underlying drivers that direct stakeholder decision-making is valuable for aligning ecological needs with societal needs. Without a SES framework, managers risk perpetuating social vulnerabilities that hamper implementation and prolong restoration, as demonstrated at Mattawoman Creek. The holistic perspective offered by a SES approach is essential for developing resilient HAB management strategies.

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