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Monitoring methods and bloom dynamic studies of the toxic dinoflagellate genus *Alexandrium*

A thesis submitted to the National University of Ireland, Galway for the degree of Doctor of Philosophy

by

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ABSTRACT

Records of annual monitoring of the dinoflagellate genus *Alexandrium* in Cork Harbour, Ireland date back to the 1990’s. Intense bloom densities in this area have caused major economical loss and impacted negatively on the ecosystem. It has been established that the noxious species *Alexandrium minutum* is responsible for the toxic events on the south coast of Ireland, leading to outbreaks of paralytic shellfish poisoning (PSP). PSP is a potentially lethal syndrome in humans caused by the ingestion of potent neurotoxins collectively known as saxitoxins, which are vectored through shellfish. A reliable method of prediction for harmful algal blooms (HABs) in this region could potentially lead to bloom mitigation methods and help reduce the severity of their impact. As we still know relatively little regarding the behavior and life cycle patterns of *A. minutum*, further investigation into its sexual and asexual reproductive phases, and how they impact upon the species’ population dynamics may assist its predictive ability. During the course of this study, *A. minutum* blooms in the North Channel area of Cork Harbour were monitored during the summer periods of 2011 and 2012. A time series data set of annual *A. minutum* bloom densities between the years 2004 and 2010 is also presented.

Hypnozygote studies carried out in situ found a mandatory cell maturation time of the order of months to precede successful excystment, with temperature acting as a significant influencing factor. Bloom magnitude was found to be independent of the cyst density measured the previous winter, suggesting hypnozygote cells provide the bloom inoculum and environmental factors determine bloom magnitude. Encystment levels within the population were also assessed through the deployment of sediment traps. It was determined that regardless of bloom intensity, an average of 2.5% of the proportion of *A. minutum* vegetative cells encyst annually within the North Channel. A cyst dynamics model simulating the annual cyst input to the sediment between 2003 and 2012 found intense blooms (>100,000 cells L⁻¹) are not necessary to maintain cyst stocks. A simple ecological model was used to simulate bloom initiation based on the environmental parameters of irradiance, temperature and tidal dilution, while sexual
reproductive stages were used to simulate bloom termination. Tidal dilution was found to exert a substantial level of control over bloom development, a process thought to require 10-14 days. It was evident that maximum bloom development followed the lowest spring tide in June. This result may contribute towards developing a reliable method of *A. minutum* bloom prediction.

Aside from the role physiological changes play in *A. minutum* population dynamics, an attempt was made to determine the level of influence parasitism exerted on the functional ecology of the population. Both parasitic marine genera *Amoebophrya* and *Parvilucifera* were indentified in the North Channel. While *Amoebophrya* was evidently infecting various dinoflagellate host species, the host to *Parvilucifera* was not confirmed. No parasitic infection was observed within the *A. minutum* population. This work constitutes the first report of infections on dinoflagellates in Irish coastal waters and creates a platform for future studies.

As observations of dinoflagellate population dynamics in the natural environment require an accurate and consistent method of monitoring, this study also researched the comparability of the various methods commonly employed for the monitoring of HAB events, and determined the potential of more automated methods. The FlowCAM showed its ability at successfully detecting individual species of interest and provided an efficient analysis of the phytoplankton community structure. No significant difference was found between it and the use of fluorescent in situ hybridisation techniques (FISH). The FlowCAM is suggested as a valuable contributor to consistent plankton records as part of ongoing monitoring efforts.
Chapter I

INTRODUCTION; background and context
1. General introduction to phytoplankton

1.1 Phytoplankton characteristics

The term phytoplankton traditionally represents the phototrophic component of all living microorganisms passively drifting within the water column, yet species of taxa such as dinoflagellates are often included in the mixotrophic and heterotrophic groups. They are found in virtually all bodies of water whether fresh, salty or brackish. The etymology of phytoplankton reveals it is of Greek origin and derived from the words planktos, meaning wanderer, and phyton, referring to plant. The habitat of these often unicellular microscopic plants is restricted to the euphotic zone in the water column as they require sunlight to photosynthesis and create chemical energy. The depth of the euphotic zone will depend on the attenuation of the sunlight, altered by the turbidity of the water column. In highly turbid fresh water systems the euphotic zone may only extend a few centimetres, yet in the open ocean it can extend beyond 100 meters. Phytoplankton also require nutrients to fuel their activity, the most essential being phosphorus, nitrogen, silicate and iron. The mixing of deep, nutrient rich waters generally during winter months provides a supply of essential nutrients to the euphotic zone. Phytoplankton proliferate when an ample supply of nutrients and irradiance is available and when the water column becomes stratified, trapping the cells nearer to the surface for maximum irradiance absorption. While nutrients can be stored by cells or recycled after absorption by an abiotic component, photons cannot, and for the phytoplankton cells to avail of its energy it needs to be converted instantly.

1.2 Importance of phytoplankton

Phytoplankton cells are invisible to the unaided eye, yet they are an invaluable component of both freshwater and marine ecosystems. They are the foundation of the food chain, and in areas of increased phytoplankton abundance, there is an increased abundance of higher trophic organisms. They serve as a critical food source for bivalve filter feeding shellfish and the larvae of crustaceans and finfish. The phytoplankton group also take responsibility as the main primary producers in the world’s oceans. By photosynthesizing, they are playing a crucial role in the carbon cycle, converting
dissolved carbon dioxide in the water column into organic matter and globally producing as much carbon in the ocean as terrestrial plants. This produces oxygen which is consequently released into the atmosphere. The main groups of phytoplankton include diatoms, dinoflagellates and cyanobacteria. There is also a diverse grouping of microflagellates which includes a group called the coccolithophores, holding significance as dimethyl sulphide (DMS) releasing organisms. When released into the atmosphere DMS acts as cloud condensation nuclei and contributes to the percentage of cloud cover. This variety of phytoplankton possesses a calcium carbonate exterior and during its construction it removes a vast amount of carbon dioxide from the atmosphere. As levels of carbon dioxide are continuing to increase in our atmosphere, phytoplankton now more than ever are an extremely important resource to help balance excessive levels of this greenhouse gas.

1. General introduction to the dinoflagellate group

1.2 Dinoflagellate characteristics

The dinoflagellates are one of the most significant and largest groups of phytoplankton, exhibiting a great diversity of form and often held responsible for the creation of exceptional blooms. The most recent classification effort of dinoflagellates estimates nearly 2,300 species are known to date, belonging to 238 different genera (Gómez 2012). While 50% of dinoflagellates are suggested to be phototrophic, gaining nutrition strictly through photosynthesis, the other 50% are thought to be able to feed through a variety of mechanisms (Taylor 2006). In addition to phototrophic, dinoflagellates can also be of a heterotrophic or mixotrophic nature, in which they are capable of ingesting other organisms and detritus as a form of nutrition. Mixotrophs combine both photosynthesis and prey ingestion. In general, photosynthetic dinoflagellate species contain chlorophylls a and c2 in addition to the accessory pigments beta-carotene, while some also contain the light harvesting carotenoid peridinin. The nuclei structure of a dinoflagellate cell is so unique it has its own term, dinokaryon, where the chromosomes are continuously condensed, and also lack protein histones. Syndinium and Noctiluca are the only two known dinoflagellate genera whose chromosomes are not permanently condensed. Aside
from the abundant free-living populations of dinoflagellates, symbiotic dinoflagellates can form a mutualistic relationship with cnidarians and can provide their coral hosts with as much as 95% of their metabolic requirements. The genus *Symbiodinium* is the most recognised out of the symbiotic dinoflagellates and is commonly found living within shallow water corals in tropical and sub-tropical regions.

1.2 Dinoflagellate taxonomy

Dinoflagellates acquired their name in 1773, with “dino” being the Greek word for whirling or turning, they are so called due to the fact they possess two flagella, which imparts a distinctive spiral motion to their swimming behaviour. The orientation of the flagella on a dinoflagellate determines whether they are a desmokont or dinokont cell type. The flagella of a desmokont cell both emerge from its anterior region (figure 1.1A). The flagella of a dinokont cell are positioned within depressed regions longitudinally in the sulcus, and transversely in the cingulum (figure 1.1B). Flagella provide the advantageous characteristic of being able to migrate freely throughout the water column. Vertical migration patterns can often be observed in dinoflagellate populations at night as they supposedly embark upon a search for nutrients (Cullen *et al.*, 1998).

Dinoflagellate vegetative cells can be one of two body forms, ‘armoured’ or ‘naked’. Both forms have a covering of flattened vesicles collectively called aveolae. In armoured cells, these vesicles fill and support a cell wall divided into organised cellulose plates known as a theca (figure 1.2). The

![Figure 1.1. Example of desmokont cell (A) and dinokont cell (B) (Source: Steidinger & Tangen 1997)](image)

![Figure 1.2. The structure of a typical *Gonyaulax* dinoflagellate cell (source: Redrawn from Fensome *et al.*, 1993)](image)
structure of the theca gives them a characteristic pattern which is often utilised in their classification. In contrast to armoured cells, naked cells are bound only by a thin pellicle. Due to the delicate nature of this membrane, the shape of the cell can easily become distorted. The armoured cells can possess up to 100 cellulose plates, creating a unique tabulation for each species. The most commonly used method for plate nomenclature in dinoflagellates is based upon a Charles Atwood Kofoid’s tabulation system (Taylor 1999). Dinoflagellates can be a variety of shapes and sizes with individual ornamentation including horns, spines and ridges. As indicated in figure 1.2, the area of the theca above the cingulum is referred to as the epitheca and the area below is referred to as the hypotheca.

1.3 Environmental parameters favouring dinoflagellate growth

While dinoflagellates are a common component of all aquatic systems, stratification of the watermass is thought to be an essential physical condition for them to proliferate and develop a bloom (Smayda 1997). Unlike diatoms, they don’t have the tolerance to succeed within a turbulent system (Estrada and Berdalet 2005). Dinoflagellate cells have been reported to increase their production of mucous during times of shear in the water column and it has been suggested that this action may be their attempt at creating a more stable physical environment (Smayda 2002). Stratification of the water column occurs when increased sunlight during the spring and summer months warm its surface. A transitional layer known as the thermocline is created between this warm layer and a cooler layer beneath, and the dinoflagellate cells are harboured above this gradient to avail of the penetrating sunlight. Diatoms generally bloom during spring, taking advantage of the high level of nutrients available after sustained mixing of the water column during winter. Diatoms generally grow at a faster rate than most dinoflagellates. During the summer months when the nutrient levels begin to fall and stratification increases due to warming temperatures, dinoflagellates will flourish, sometimes creating high density blooms. A single species can often dominate the dinoflagellate community. Due to the competitive exploitation of resources, mono-specific dinoflagellate blooms may cause a diminution in phytoplankton diversity within the affected region and can alter an established system of biotic interactions.
1.4 Lifecycle of dinoflagellates

The life cycle of dinoflagellates can be quite complex, differing in motility, physiology and life styles. A non-motile benthic stage is common within the marine dinoflagellate group, with more than 80 species known to encyst (Matsuoka et al., 2003). There are two possible stages of dormancy, a temporary cyst and a resting cyst. A temporary cyst has lost its flagella in a response to shock, either mechanically induced or resulting from lengthy exposure to stressful conditions. It can take refuge in the sediment, but will re-establish motility once the surrounding environmental conditions are restored. The ability to recover quickly is an important feature of temporary cysts. A resting cyst, or zygote, is formed during sexual reproduction. Planktonic zygotes may swim for several days before sinking to the sea or lake floor. The germination of these seeds will not take place until conditions of temperature, light and nutrient availability become favourable. Cysts can remain viable in sediments for several years (Kelly 1993). It has also been suggested that the germination of resting cysts can be controlled by an internal mechanism similar to a biological clock (Anderson and Keafer, 1987). Encystment rates, dormancy length and germination rates can be species or even strain specific (Anderson 1998).

2. An introduction to harmful algal blooms

2.1 Determination of harmful algal blooms

In most cases, the proliferation of microscopic algae within a water body is of benefit to the overall ecosystem, providing a food source for organisms at the lower end of the food chain. However, algal blooms which have a negative impact on their surrounding environment occur globally within both freshwater and marine habitats and are referred to as harmful algal blooms (HABs). The first documentation of a HAB event dates back to biblical times and quotes ‘all the waters that were in the river turned to blood, and the fish that was in the river died’ (Exodus 7: 20-21). This literature is referring to the occurrence of a red tide; a colloquial name often used to broadly describe a HAB event. This term is not entirely accurate as the majority of HABs are in fact colorless and unrelated to tidal movements. The most common HAB genera which cause discoloration of the water due to their pigments are *Karenia*, *Noctiluca* and *Lingulodinium*. HABs are a
natural phenomenon most often observed in coastal regions. The two main processes by which a HAB event can negatively impact upon its ecosystem are oxygen depletion resulting from intense cell concentrations and the synthesis of toxins by particular dinoflagellate species. When a high density bloom starts to decay, increased respiration of bacterial organisms can induce anoxic conditions lethal to surrounding marine life. Increased respiration levels of algae during times of low irradiance are also a contributor to oxygen depletion. In contrast to oxygen depletion, increased cell proliferation can also cause oxygen super saturation of surface waters. Values of 160% have been recorded in surface waters which may impact negatively on surrounding fish populations, as large oxygen bubbles can irritate their gills (Purdie 1996). The toxins produced by a number of dinoflagellate species can induce devastating effects on marine life, and if ingested by humans the result can be fatal. The most common vector of these toxins to humans is through the consumption of contaminated filter-feeding shellfish. Toxic blooms are generally mono-specific, i.e. only involve one species. This species may not have the highest biomass within the plankton community, yet it can create significant harm (Richardson 1997). Out of all the dinoflagellate species known to date ~ 5% of them are toxin producers and found within marine habitats (Steidinger et al., 2011).

2.1.2 Environmental and economic impacts
The occurrence of HABs can impart severe consequences on the surrounding environment and affect the general health of the ecosystem. As mentioned above, bivalve filter-feeding shellfish often harbor toxins during HAB events. In many cases the shellfish are unaffected by these toxins and act only as a vector, yet they can also fall victim to their potency. For example, shellfish starvation can occur when toxic algal species dominate within the phytoplankton community (Smayda 1997). This may be as a result of the harmful algae interfering with the digestion system of the organism or it may simply have distaste for the algae species (Gallagher et al., 1989; Wikfors and Smolowitz 1995). Mass fish kills are not an uncommon event during HABs, whether due to anoxia or as a result of toxins, e.g. brevetoxin. Spine forming diatoms are also known to cause the mortality of fish stocks by becoming trapped in the gills of the fish, resulting in mucous accumulation, respiratory failure, and bacterial infections (Rensel 1993).
addition to shellfish and finfish, higher trophic levels can also fall victim to the affect of HABs, including some of the oceans largest mammals. The mass mortality of sea lions, manatees and cetaceans induced by toxin producing algae has been observed in the past (Geraci et al., 1989; Bossart et al., 1998; Scholin et al., 2000).

One of the first recorded cases of human mortality resulting from the consumption of toxic shellfish dates back to 1793 in British Columbia when the crew of Captain George Vancouver ingested contaminated clams from an area now known as Poison Cove. Although an increased awareness of shellfish poisoning exists today, there are on average 2000 cases annually of humans falling ill after consuming contaminated shellfish and at least 300 of these cases are thought to end in fatalities (Hallegraeff et al., 2003). A range of syndromes can result from shellfish toxicity including diarrheic shellfish poisoning (DSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP) and azaspiracid shellfish poisoning (AZP). With the exception of ASP which arises from the diatom genus *Pseudonitzschia*, all syndromes are induced by toxic dinoflagellates. The bio-accumulation of toxins within shellfish can result in huge economic loss for shellfish industries. In order to comply with the European Council Directive 91/492, harvesting is no longer permitted in an area where toxin concentrations in the water/flesh of the shellfish exceed a threshold level. A report by Hoagland et al., (2002) estimates HAB events to cost the United States in excess of 50 million per annum. Yet it has more recently been suggested that the overall economic impact HABs incur upon the aquaculture industry is not yet known in its entirety and increased measures need to be taken to create a reliable method of estimation (Hoagland and Scatasta 2006).

2.1.3 Bloom prediction and mitigation

HAB events are the natural result of a combination of biological, chemical and physical parameters favoring cell growth. Because bloom development is not reliant on any one environmental condition, a prediction is often difficult. The global expansion of HABs in recent decades (Hallegraeff 1993) and an increased awareness of their impact have called for the need to develop early warning systems forecasting the onset of bloom periods.
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The serious risk posed to public health during a HAB event requires robust estimates of their timing and initiation. This would also enable the better management of HAB impacts. Forecasting systems such as ‘HAB-OFS’ run by NOAA and ‘ASIMUTH’ run by the Irish Marine Institute/ DOMMRS, deliver short term HAB alerts for the Gulf of Mexico and European Atlantic regions respectively. Coastal blooms which originate off-shore may be slightly easier to predict than those which initiate near shore. Off shore populations tend to rely on favorable hydrographical and meteorological conditions to transport them nearer to shore, as illustrated in Raine et al., (1993, 2010). In these two case studies, a five day weather forecast allowed for the reasonable prediction of Karenia and Dinophysis sp. blooms along the south-west coast of Ireland. A recent study by Wyatt and Zingone (2013) highlights the importance of paying closer attention to life history phases in order to assist the predictive ability of HAB forming species. Life cycles patterns can play a significant role in the both bloom initiation and bloom termination.

The reliable prediction of blooms is required to develop effective methods of mitigation and minimize the negative impacts of HABs on our resources. Methods of mitigation tested in the past include dusting copper over a bloom of Karenia brevis (Rounsefell and Evans 1958), the spreading of clay to flocculate and sink algal cells (Sengco and Anderson 2005) and the skimming of surface water to aggregate and remove cells from the water column (Shirota 1989). The introduction of marine parasite species to a water body has been long suggested as a potential biological control of HABs (Taylor et al., 1968; Salomon et al., 2003; Chambouvet et al., 2008). Once a cell becomes infected it has little chance of survival. This may be the most natural way of suppressing HAB events and the species specific nature of the parasitic genus Amoebophrya may allow for the protection of other organisms in the ecosystem. Parvilucifera and Amoebophrya are the two most commonly observed parasitic marine flagellates known to infect dinoflagellate populations. Although the specificity of Parvilucifera is questionable (Lepelletier et al., 2013) it has shown its ability to infect dinoflagellate populations (Norén et al., 1999; Garcés and Hoppenrath 2010). Amoebophrya, the more commonly studied genus, has been noted to reach relatively high prevalence rates of 40-80% within
host dinoflagellate populations (Cachon 1964; Coats et al., 1996; Maranda 2001; Chambouvet et al., 2008). This suggested method of mitigation requires further investigation before being tested in the environment.

2.1.4 Anthropogenic influences on ‘seed’ dispersal

As already mentioned, many dinoflagellate species have the ability to encyst overwinter and germinate the following year when favorable environmental conditions return. This strategy often provides the inoculation for recurring annual blooms. A global expansion in the distribution of dinoflagellate species along coastal regions, many of them harmful, may be a result of resting cysts being transported anthropogenically (Hallegraeff 1993). Ballast water has been frequently suggested as a cause of cyst dispersal, extending the geographical range of non-indigenous species (Hallegraeff 1991). There is evidence to suggest that the ballast water of an ocean vessel was responsible for the introduction of harmful dinoflagellate species into areas of aquaculture in Australia (Hallegraeff and Bolch 1992). Unless the species being transported is of a heterotrophic or mixotrophic nature, vegetative cells will most likely not survive extended journeys within ballast tanks due to the dark environment. Resting cysts on the other hand are capable of surviving adverse conditions for long periods of time (Keafer et al., 1992), and upon the release of ballast water may take up residence in previously uninhabited sediment. It has been reported that the size of the inoculum does not indicate the magnitude of the bloom (Cosgrove et al., 2014), so essentially only a few cysts can result in a substantial impact. To reduce the risk of potentially contaminated ballast water disposal within HAB free environments, it would be advisable for ballast tanks not to be filled in port during a HAB event, filled in deeper waters and not released near areas of aquaculture. Yet these proposals are not always practical. The translocation of shellfish stocks may also result in the transfer of harmful cysts as they can be retained within and released from the digestive tracts of the filter-feeding bivalves (Scarret et al., 1993). However they are transported, local scale dispersal of toxin producing dinoflagellates will no doubt continue to contribute to the increasing geographic distribution of HAB events.
3. The dinoflagellate genus *Alexandrium*

3.1 Classification and taxonomy

*Alexandrium* is perhaps one of the most studied harmful algal genera worldwide and one of the most important due to its distribution and the severity of its bloom consequences. The first species of *Alexandrium* was documented in 1904 by Paulsen and characterized as *Goniodinium ostenfeldii*. The genus *Alexandrium* was then created in 1960 by Halim, based on the single species we now know as *Alexandrium minutum*, first isolated from Alexandria harbor in Egypt. Multiple changes to its nomenclature have altered the classification of *Alexandrium* over the last century (Balech 1995). Table 1.1 illustrates its current classification. Over 30 species of *Alexandrium* are known to date and at least 15 are thought to be harmful (Anderson *et al.*, 2012).

Table 1.1  
Current classification of the genus *Alexandrium*

<table>
<thead>
<tr>
<th>Domain</th>
<th>Whittaker and Margulis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Haeckel 1866</td>
</tr>
<tr>
<td>Phylum</td>
<td>Cavalier-Smith 1991</td>
</tr>
<tr>
<td>Division</td>
<td>(Butschli) Fensome <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Subdivision</td>
<td>(Fensome <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Class</td>
<td>Pascher 1919</td>
</tr>
<tr>
<td>Subclass</td>
<td>(Fensome <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Order</td>
<td>Taylor 1980</td>
</tr>
<tr>
<td>Suborder</td>
<td>(Fensome <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Family</td>
<td>Lindermann 1928</td>
</tr>
<tr>
<td>Subfamily</td>
<td>(Fensome <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>Genus</td>
<td>Halim 1960</td>
</tr>
</tbody>
</table>

*Alexandrium* spp. cells all possess a theca, range in size from 20-80 μm and are of the dinokont variation. Compared to the morphological traits of some dinoflagellate genera
which exhibit varying ornamentation, *Alexandrium* species are more or less homogeneous in their appearance. The most prevalent features of *Alexandrium* used for taxonomic purposes and to distinguish between species include the presence/size of a ventral pore located on the first apical plate, whether or not the first apical plate is connected to the apical pore complex and the shape of both the sixth precingular and first apical plates (Hoppenrath *et al.*, 2009). Staining of the theca is required to observe these features under light microscopy. The tabulation pattern of a typical *A. minutum* cell is illustrated in figure 3.1, highlighting the significant features in ventral view. A feature commonly used to identify *A. minutum* is the sixth precingular plate (6‘‘) being more high than broad.

3.2 Geographical distribution

*Alexandrium* is a cosmopolitan genus distributed globally from the Polar Regions to temperature and tropical waters (Taylor *et al.*, 1995; Lim and Ogata 2005; Zhang *et al.*, 2007), and it can be found within a variety of oceanic systems. Although biogeographical studies have highlighted the global distribution of *Alexandrium* over the last few decades, it is thought many regions still lack comprehensive data (Anderson *et al.*, 2012). While in other areas, what appears to be the increased dispersal and diversity of *Alexandrium* populations may in fact be simply the result of a higher level of research and observation. The Mediterranean region may be an example of this, where at least twelve distinct species of *Alexandrium* have been found inhabiting Mediterranean waters to date (Penna *et al.*, 2008). The distributions of toxic *Alexandrium* species appear to be documented more than the non-toxic species, possibly due to the increased awareness concerning their harmful impacts. Studies which have successfully analyzed both toxic and non-toxin populations of *Alexandrium* along a coastline have found they are generally geographically isolated (Touzet *et al.*, 2008, 2010, Brosnahan *et al.*, 2010). In addition to increased monitoring, Anderson *et al.*, (1989) suggests three other possibilities for the
observed expansion of *Alexandrium* populations. These include the natural dispersion of cells as a result of oceanic currents, changing environmental and meteorological conditions potentially favoring bloom growth and the anthropogenic dispersal of cells.

3.3 Paralytic Shellfish Poisoning

*Alexandrium* is one of the main synthesizers of 24 potent neurotoxins (Luckas *et al.*, 2005) which vary in stability and toxicity yet accumulatively can induce paralytic shellfish poisoning (PSP). The paralytic shellfish toxin (PST) suite includes the potent saxitoxin, a collection of heterocyclic guanidines and possibly the most lethal of all the PSTs. It was the first metabolite of its kind not only to be isolated, but also to have an origin traced to plankton (Daranas *et al.*, 2001). The symptoms of PSP are purely neurological and block the neuronal and muscular sodium channels, restricting signal transmission. Mild symptoms include tingling and dizziness, while more serious cases affect the respiratory system and cause paralysis. If left untreated, PSP can lead to death within 24 hours (Halstead and Scantz 1984). There is no known antidote for PSP (Dale *et al.*, 1987) and treatment requires a gastric pump and artificial respiration. Consistent and reliable monitoring methods are required in areas of aquaculture which have a history of PSP outbreaks. Figure 3.2 illustrates all known areas worldwide to be affected by PSP by the year 2006. The detection of PSTs above a designated threshold level should result in the instantaneous closure of the harvesting site (Shumway *et al.*, 1995). The regulatory limit of PSTs typically held for shellfish flesh is 80 µg saxitoxin/ 100 g tissue. Various analytical methods have been developed to detect PSTs over the last few decades and

![Figure 3.2. The global distribution of PSP outbreaks by 2006 (source; National office for HABs, WHOI)](image-url)
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high performance liquid chromatography (HPLC) now replaces the mouse bioassay as the most commonly used technique.

3.4 Life history phases

The complete life cycle of *Alexandrium* involves five main phases as illustrated in figure 3.3 and all life stages vary in their morphological and physiological characteristics. *Alexandrium* is a haplontic, meroplanktonic genus with both motile and non-motile (cyst), haploid and diploid stages. These include a vegetative, haploid gamete, diploid zygote (planozygote), resting cyst (hypnozygote) and diploid planomeiocyte phase. A sixth phase consists of a haploid pellicle cyst. The population dynamics of *Alexandrium* is greatly influenced by its various life stages and the bloom inoculum is often provided by cyst germination. *Alexandrium* cells are not generally observed in the water column for extended periods as their life span tends to be short (Wyatt and Jenkinson 1997). Yet during exponential growth they can reach densities of $10^6$ cells L$^{-1}$ (Cosgrove *et al.*, 2014) and impact greatly on their surrounding environment. Life stage transitions are usually observed in situ under laboratory conditions due to the difficulties presented with field observations. A real challenge comes with trying to reconstruct ever changing environmental parameters in situ, and so laboratory studies run the risk of not always providing an accurate representation of cell behavior when in the environment. Nonetheless, in situ experimentation does provide insight into the life cycle of *Alexandrium* and often reveals gaps within our current knowledge.

Figure 3.3. Life history phases of *Alexandrium*. 1. germination 2. Planomeiocyte formation 3. Asexual division producing haploid daughter cells 4. Gametogenesis 5. Diploid zygote. (Source Jack Cook, WHOI)
3.4.1 The vegetative phase
In the vegetative state, *Alexandrium* species reproduce asexually by means of binary fission and the daughter cell usually retains one half of the parent’s theca indicating desmoschisis (Figueroa *et al.*, 2008). A maximum rate of asexual division is believed to occur during defined intervals such as the period from darkness to light (Chisholm 1981). The length of time cells spend in their vegetative state predominantly depends on the surrounding environmental parameters and is generally only a matter of weeks. It is a long established fact that during their vegetative phase, *Alexandrium* cells tend to aggregate towards the surface of the water column (Rasmussen and Richardson 1989). Diel vertical migratory behavior has also been observed in natural populations (Anderson and Stolzenbach 1985). The traditional view is that vertical migration activity may be employed by the cells to access pools of essential nutrients in the water column during times of low nutrient concentrations at the surface. Aside from division, life stage transitions which occur during the vegetative stage include pellicle cyst and chain formation. Pellicle (temporary) cysts are non-motile and posses a thin cell membrane. Their formation is often a reaction to unfavorable environmental conditions, essentially a strategy undertaken by the cell to ensure survival. Grazing and parasites may also influence the onset of this transition (Anderson *et al.*, 2013). The average swimming speed of an *Alexandrium* cell is thought to be 1 m h⁻¹ (Eppley *et al.*, 1968), yet the formation of cell chains, a characteristic observed in the vegetative state of some species including *A. catenella* and *A. affine* can increase the swimming speed (Fraga *et al.*, 1989). Chain formation is observed more frequently within natural samples than in culture, suggesting it may be a reaction to turbulence (Sullivan *et al.*, 2003) and a method employed to help resist cell dispersion.

3.4.2 The sexual phase
Gametogenesis is a poorly understood life transition of *Alexandrium*. It has been suggested that once a population reaches a threshold cell density, gametogenesis begins (Wyatt and Jenkinson 1997). The reduced nearest neighbour distance between cells increases the chance of collision and in turn, the successful fusion of gametes cells. Studies have also suggested that nutrient deficiency is responsible for the onset of
sexuality in *Alexandrium* (Probert *et al.*, 2002; Figueroa *et al.*, 2007). Determining the initiation of sexual reproduction in a population often presents difficulties due to the lack of differentiation existing between vegetative and gamete cells. Some reports have suggested dinoflagellate gamete cells are smaller than the vegetative equivalent (Von Stosch 1973; Kremp and Heiskanen 1999), yet Figueroa *et al.*, (2007) found virtually no difference between *Alexandrium* gamete and vegetative cells, deeming them to be homologous in their morphology. The first stage of sexual reproduction in *Alexandrium* which can be morphologically differentiated from the asexual stage is the formation of the planozygote cell. A planozygote is generally a larger and darker cell with a more granulated appearance, making it potentially identifiable using light microscopy. They also possess two trailing flagella, yet this feature is not always clearly visible and can be affected by preservation techniques. Although it was once thought planozygotes could not revert back to a vegetative state, a recent study by Figueroa *et al.*, (2006) observed the division of *Alexandrium* planozygote cells when placed in nutrient replete medium. Cell concentration may also play a role in deciding the sexual ‘faith’ of a planozygote cell (Uchida *et al.*, 2001). *Alexandrium* spp. may be of a homothallic or heterothallic nature, where sexuality can be induced in a single clonal culture or require two anisogamous mating types (+/-). A complex heterothallic mating type has also been identified within populations of *A. minutum* and *A. tamutum* suggesting more than two sexual types are required for cell conjugation (Figueroa *et al.*, 2007). A lot still remains unknown about how gametes actually manage to locate each other in the water column. Wyatt and Jenkinson (1997) suggest that in many cases, increased cell concentrations alone will not suffice for gamete collision and proposed the production of pheromones as a method of cell recognition.

3.4.3 Encystment
Planozygote cells which progress to encystment are thought to do so within 7 days (Anderson 1998). They enter a non-motile phase after shedding their theca and accumulate on the sea bed. Mud flats provide the ideal habitat for retaining cyst populations as both extra fine and extra course grain sediment can result in cyst dispersal during turbulence. A typical *Alexandrium* cyst possesses a tough cyst wall encompassing
storage products to ensure survival over winter. The main features used to distinguish *Alexandrium* cysts, as described by Matsuoka and Fukuyo (2000), are its shape (often spherical to ellipsoidal), lack of ornamentation, color of cell wall (colorless) and its archeopyle type (mostly saphopylic or precingular). Toxic seedbeds can prove to be very destructive in an ecosystem, with cysts reported to be up to ten times more toxic than the vegetative cell (Dale *et al*., 1978). They have the ability to survive in unfavourable conditions and continue to infect inhabitants in the absence of the vegetative phase. Resting cyst formation also supports genetic recombination in a population as they are compositions of multiple generations of planktonic vegetative cells. As a result, cyst beds which essentially act as genetic repositories ensure both the successful survival and persistence of a population in an area over time.

3.4.4 Excystment

Resting cysts have been documented to survive for extended periods of time within the sediment (Keafer *et al*., 1992) even under anoxic conditions. Oxygen is essential for their germination, so cysts residing in lower sediments rely on resuspension or bioturbation efforts to reach the oxygenated layer. Temperature has also been illustrated as a factor influencing excystment (Anderson 1980; Pfiester and Anderson 1987). Even when optimum conditions are presented, it is thought dinoflagellate cysts will not excyst before completing a mandatory dormancy period. This can vary depending on the species. For example, Figueroa *et al*., 2007 found varying mandatory dormancy periods between two *Alexandrium* species, *A. tamutum* and *A. minutum*. The length of time cells remain quiescent may impact their bloom strategy, as those which require an order of months will most likely overwinter as a resting cyst, while those which can excyst in a matter of weeks can switch between vegetative and benthic phases more efficiently. The existence of an internal biological clock controlling the timing of germination has been demonstrated within *A. fundyense* cells in the Gulf of Maine (Anderson and Keafer 1987). Yet, it appears to influence the deeper coastal populations as oppose to those found in shallower regions (Anderson 1997). It may be the case that bottom waters need to rely on an internal cue if environmental parameters fail to cause the sufficient impact
required to drive seasonality. A seasonal characteristic has also been observed in *Alexandrium* cysts isolated from Cork Harbour (Ní Rathaille and Raine 2011).

4. Species identification and monitoring methods

4.1 Choosing a monitoring method

For successful bloom monitoring, a program has to be designed relevant to the geographic interest. As stated by Lund *et al.*, (1958), there is no one method for algal monitoring which best suits every situation. It is important to start with a defined objective and have a firm expectation of the level of insight the effort will bring. Monitoring can be used for observational, predictive or quantitative analysis and the method which best compliments each category needs to be carefully chosen. If one requires information regarding long-term changes in the ecosystem, a reliable method which is not susceptible to observer bias is needed. For short term observations a rapid yet accurate method is of more benefit as it allows for the efficient analysis of an increased number of samples. The time required for sample processing and analysis should also be taken into consideration when choosing a method, as although the analysis of the sample may be quick, the process involved in its preparation may be lengthy. In addition, the cost of the method is important, especially if the analysis of a high number of samples is required.

4.1.2 Traditional methods

Many of the more traditional methods used for qualitative and quantitative analysis of phytoplankton samples involve light microscopy. One of the original and possibly most reliable methods of algal cell quantification is the Utermöhl method first developed in 1931 and which to this day has hardly been modified. The Utermöhl method is often chosen as the standard method of estimating phytoplankton abundance, allowing results from various locations to be compared. A sub-sample of up to 50 mL is left to settle for several hours within a chamber, the base of which comprises a thin glass plate. This sample is then examined by light microscopy. This results in a delay of up to 24 hrs before analysis and is the main disadvantage associated with an otherwise relatively
flawless method. When analyzing samples containing toxin producing species such as *Alexandrium* spp. and public health is potentially at risk, this method may be too time consuming. Yet, a study by Paxinos and Mitchell (2000) found the application of a plunger effect to the Utermöhl method reduced processing time from 24 hrs to 2 hrs, yielding similarly accurate results. The settlement bottle technique as described by McDermott and Raine (2010) is based on the same principle as the Utermöhl method, but no errors arising from manipulation of the samples are introduced. The samples in cell culture bottles can also be directly archived for reanalysis at a later date. Although it has its limitations, microscopy is an invaluable tool in phytoplankton assessment and provides opportunity for the identification, enumeration and relative abundance of algal species within a community, as well as the determination of various life stages, including resting cyst and zygote formation.

4.1.3 Automated methods

In recent times, the development of automated methods allowing for cell quantification and identification has created a more efficient and less tedious way of analysing phytoplankton samples. Automated analysis may also contribute greatly to monitoring programs which require the processing of a high volume of samples over extended periods of time and potentially reduce observer bias. The first recognized advance towards the automated quantification of algal cells was the development of the Coulter Counter during the late 1960’s (Sheldon and Parsons 1967). Cell concentrations were estimated based on the voltage signals created by particles passing within a medium. Flow cytometry methods overshadowed the use of the Coulter Counter during the 1980’s as it allowed for the distinction between cells and detritus. In 1981, flow cytometry was used to determine saxitoxin content within *Alexandrium* sp. cells and has since been employed for the rapid quantification of microalgae cells (Veldhuis and Kraay 2000). The method is based upon light scatter and fluorescence measurements at varying wavelengths. Further advances in automated monitoring methods in more recent years include the development of an environmental sample processor (ESP) (Scholin *et al.*, 2009). A moored ESP relays data in real time while identifying and enumerating species though the use of molecular assays, as well as the discrimination of their chemical
components. This complex instrument intends to act as an early warning system for toxic bloom events and is currently used to detect *A. fundyense* species in the Bay of Fundy. The main advantages of an automated method over a manual method is the fact they are often less labor intensive and can process a larger amount of samples with perhaps greater sensitivity and increase statistical significance. The restrictions presented by automated methods include the high cost of the instrument, its often rigorous maintenance and in many cases upper and lower size limitations.

4.2 Molecular methodologies
Both traditional and automated methods have been successfully used in the past to detect and enumerate *Alexandrium* species within discrete water samples. But *Alexandrium* species generally have homologous features, and so distinguishing between them can be difficult when the method of identification used is based upon morphological traits. Developments in molecular microbial ecology over the last two decades have allowed the discrimination between morphologically similar yet genetically distinct species. Utilizing the unique genetic structure of a species, molecular methods allow the rapid, sensitive and reliable detection of target cells within mixed community samples. Deoxyribonucleic (DNA) and ribonucleic (RNA) acids store all the species specific genetic information within an organism. In eukaryotes, ribosomal DNA (rDNA) is a sequence which codes for ribosomal RNA (rRNA). The rDNA operon (figure 4.1) is composed of an 18S small subunit (SSU), 28S large subunit (LSU), internal transcribed spacers (ITS1 and ITS2) and 5.8S gene (a non-coding RNA component). Non-transcribed spacers (NTS) and external transcribed spacers (ETS) separate the genes. The alignment of rDNA sequences can provide extensive information on phylogenetic relationships, while both rDNA and rRNA have gained much attention in recent year due to the development of specific gene probes capable of targeting these molecules and discriminating between species. The classification of species, especially morphospecies has greatly benefited from the development of molecular tools and probes.
4.2.1 Polymerase Chain Reaction
The development of Polymerase Chain Reaction (PCR) techniques in phytoplankton ecology has made a major contribution to the classification and phylogeny of phytoplankton groups. Its application has revealed extensive information regarding the geographic dispersal and taxonomic history of *A. minutum* (Lilly *et al.*, 2005). This method replicates and amplifies nucleic acid sequences and uses primers composed of artificial DNA strands which compliment the sequence of the target DNA. A program specific to each reaction is set on a thermo-cycler which consists usually of 20-25 cycles and four main steps. These steps include denaturation (separation of DNA), annealing (binding of DNA to primer sequence), elongation (amplification of target DNA by polymerase) and final elongation (ensuring all DNA strands are copied). To ensure the correct DNA was synthesized, results are analyzed under UV illumination on an agarose gel platform. The determination of the nucleotide sequence of the target DNA is achieved by DNA sequencing. This involves the replication of the target DNA using DNA polymerase, which catalyses the insertion of nucleotides to their correct bases. Original sequencing methods are based on the chain termination method as described by Sanger and Coulson (1975), but a variety of next generation sequencing methods are under development to lower cost and increase efficiency.

4.2.2 Fluorescent In Situ Hybridization
Fluorescent in situ hybridization (FISH) is a cytogenetic technique used for specific species detection since the 1980’s. Fluorescent oligonucleotide probes are used to bind to the complimentary sequences of rRNA in the ribosome material and highlight the target cell. This method has been successfully used for the detection of harmful algal species
including *Alexandrium* spp. (Sako *et al.*, 2004; John *et al.*, 2005; Kim *et al.*, 2005; Touzet *et al.*, 2009). During periods of low cell concentration in the water, increased volumes of 2-3 L can be concentrated and analyzed using FISH. This increases the accuracy of the cell concentration estimate compared to some of the more traditional methods mentioned earlier. For the hybridization of the probe to the cells, the sample is concentrated on a membrane filter. Epifluorescence microscopy is then used for the quantification of the target cells. Due to the possibility of cell aggregation, it is advised the entire filter is analyzed to account for all material. Catalyzed reporter deposition FISH (CARDFISH) is a more powerful technique based on tyramide signal amplification and employed in recent years to quantify microbial populations with reduced signal intensity. The application of CARDFISH to HABs has predominantly been used for the detection of bacterial activity and marine parasites including *Amoebophrya* spp. (Garcés *et al.*, 2007; Chambouvet *et al.*, 2008).

**5. *Alexandrium* spp. in Irish waters**

**5.1 Distribution**

Several *Alexandrium* species have been identified along the southern and western coastlines of Ireland. They include *A. minutum*, *A. tamarense*, *A. ostenfeldii*, *A. andersoni*, *A. tamutum* and *A. peruvianum* (Touzet *et al.*, 2008). A study by Touzet *et al.*, (2007) also found the populations of *A. minutum* on the southwest coast to be toxic while strains of *A. minutum* isolated from the west coast were non-toxic. Cluster analysis following the Random Amplification of Polymorphic DNA (RAPD), found both populations clustered individually from each other, yet no genetic dissimilarity was determined in the LSU or ITS regions of the rDNA (Touzet *et al.*, 2007). The majority of studies concerning *Alexandrium* in Ireland have predominantly focused on the south coast due to the presence of toxic *A. minutum* populations. There is an invested interest in monitoring HAB events in this region as it boosts an extensive aquaculture industry. Seasonal surveys analyzing the distribution of *A. minutum* cyst ‘seed’ beds have also been conducted in an attempt to link vegetative bloom dynamics to its benthic stage (Ni Rathaille and Raine 2007; Cosgrove *et al.*, 2014). An area known as the North Channel in
Chapter I

Cork Harbour contains multiple *Alexandrium* cyst bed hotspots as a result of its retentive hydrographic features and fine mud sediment.

5.2 Modeling bloom dynamics

The prediction of *Alexandrium* bloom events along the south coast is highly desirable, in order to reduce their impact on the general ecosystem and areas of aquaculture cultivation. Knowledge regarding bloom timing and length would help alleviate both economic loss and the risk to public health. A previous study by Ní Rathaille (2007) used mathematical modeling to simulate the initiation of an *Alexandrium* bloom in the region of Cork Harbour based on ecological and hydrological parameters. A simple one-dimensional box model was developed based on the influence of excystment, tidal dilution, temperature and irradiance. It was hypothesized that these parameters alone could be responsible for *Alexandrium* bloom dynamics observed within the Cork Harbour area. This was based on a hypothesis by Ryther (1995) which states that the hydrographic and meteorological conditions required for cell growth and accumulation alone are needed to permit a HAB event. The main results from the Cork Harbour study conclude that a temperature of 15 °C, Irradiance > 100 µE ·m⁻² ·s⁻¹ and periods of lower tidal dilution encourage *Alexandrium* bloom development in the Cork Harbor area. The seasonal excystment pattern was found to contribute to bloom growth during late May and June, while nutrients were not important in growth limitation. The study pioneered for further investigations into the dominant factors influencing the growth of *Alexandrium* spp. and the role of life history stages on bloom dynamics.

It is essential for management purposes that we have an understanding of the main factors which control HAB events. Modeling is a suitable method for investigating various processes, evaluating their relative importance and discovering gaps within our knowledge. By describing HAB events on local hydrographic and meteorological conditions will often help define a period of time during which bloom events may be more likely to occur. When research focuses on a single influencing parameter, the interaction between physical and biological processes are often not addressed. Revealing the interactions between these processes helps to create a more complete, integrated
picture of what is happening in the environment. Modeling studies have been successfully used in the past to gain insight into the population dynamics of *Alexandrium* spp. based upon a coupled physical-biological model (Mc Gillicuddy *et al.*, 2005; Stock *et al.*, 2005; Fauchot *et al.*, 2008).

**Chapter summary**

The review in this chapter provides an introduction to the various topics and methodologies which are explored further over the next five chapters. It has documented the main biological, ecological and physiological characteristics of phytoplankton, dinoflagellates and the genus *Alexandrium*. It has outlined monitoring methods used for the enumeration and identification of phytoplankton, the molecular methods advancing species quantification and discrimination, the potential of marine parasites to act as a biological control for HAB events, and the role of mathematical modeling in *Alexandrium* population dynamics and bloom prediction.

Chapter II represents an investigation into the various methods commonly employed for enumerating *Alexandrium* spp. within heterogeneous samples. The ability of an imaging flow cytometer (FlowCAM) is assessed and compared to both microscopy and molecular techniques used for the quantification of cells. Long term monitoring efforts of HAB events require accurate, efficient and non-bias methods of analysis. For this reason, it is likely more automated/semi-automated protocols will be applied within global monitoring programs in the future, increasing the volume of sample processing and potentially lowering observer bias over time. The main objectives of this chapter include: outlining the advantages and disadvantages of the Utermöhl method, FISH, and the FlowCAM for the quantification of *A. catenella* cells measured from Santa Cruz Wharf, California; an area which requires year round monitoring, and also exploring the use of oligonucleotide probes to increase the species specificity of automated methods. The coupling of automated methods with specific probes is a relatively recent development in HAB monitoring. It is one which holds great potential, but requires further research to establish its reliability.
Chapter III is a novel study on the Irish coastline, exploring the potential of marine parasites to act as a biological control within dinoflagellate populations. The main aim of this research is to determine whether the toxic *A. minutum* population within Cork Harbour is infected by either marine parasitic genera *Amoebophyra* or *Parvilucifera*, and if so, to what extent. Although algal parasites have long been proposed as a biological control of HABs (Taylor 1968), little is known regarding their dynamics and ability to suppress a bloom in the environment. In this chapter we use molecular technology to determine and characterize the presence of marine parasites within the North Channel environment, and explore the level of control they exert over the population dynamics of their hosts. Although first discussed 46 years previous, this area of research has received most interest within the last decade. Yet, few studies have focused on the species *A. minutum* with regard to parasitic infection.

Chapter IV and V deal with *A. minutum* life-history stages and the influence of both the asexual and sexual reproductive stages on population dynamics. Chapter IV focuses predominantly on the encystment phase. One of the main aims of this study is to explore the theory of a cell density threshold being required for successful gamete collision and fusion, as suggested by Wyatt and Jenkinson 1997. To date, literature on this topic is limited. The relationship between cyst density and bloom magnitude is also assessed, as it has previously been suggested that cyst densities can influence bloom density (He *et al.* 2008; Cosgrove *et al.*, 2010). Chapter V focuses on the earlier stages of the sexual reproductive cycle including gameteogenesis and planozygote formation. As these stages are morphologically more difficult to identity, little work has been carried out on their dynamics. The aim of this chapter is to estimate the proportion of the vegetative population which progress to planozygote cells and in turn, the proportion of planozygote cells which progress to encystment. An ecological model is used with the intention of simulating bloom initiation based on varying environmental factors, and bloom termination based on sexual reproduction. The objective is to hind cast the result against the observed bloom densities and determine the main influencing factors on *A. minutum* bloom dynamics.
The final chapter VI comprises a short study examining the density and distribution of *Alexandrium* resting cysts within the North Channel. This chapter is a conference proceedings paper, presented in 2010. The aim of this work is to determine whether a relationship exists between *A. minutum* cyst densities and cell densities utilizing an eight year dataset from the Cork Harbour area. The areas of highest density are outlined and an attempt is made to relate these findings to hydrodynamic activity.

The overall aim of this thesis study is to gain further insight into the genus *Alexandrium*. Potentially toxic species of this genus cause harm on a global basis and increased knowledge of their life cycle dynamics and behavior is essential to aid future methods of prediction. An assessment of the life-history stages, bloom dynamics and persistence of *A. minutum* is presented in the following chapters, in addition to a review of dinoflagellate parasitic activity within Cork Harbour, Ireland and an assessment of the capability of a FlowCAM as an automated method of bloom monitoring.
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Chapter II

The quantitative analysis of *Alexandrium catenella* in Monterey Bay, California using imaging flow cytometry

Planned submission for Harmful Algae

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ABSTRACT

Harmful algal blooms pose a threat to the health of both humans and surrounding marine life and require consistent and reliable methods of monitoring. Current methods of monitoring including light microscopy and fluorescent in situ hybridisation (FISH) can be time-consuming. The FlowCAM, an imaging flow cytometer, provides a rapid, automated and economical method of analyzing phytoplankton communities within natural samples. We investigated the ability of the FlowCAM to identify and enumerate *Alexandrium catenella* cells within natural samples collected weekly from the Santa Cruz Municipal Wharf. Cell concentrations recorded using both the FlowCAM and more traditional methods of enumeration were compared over an eight week period. No significant difference was found between the quantitative analysis of *A. catenella* using FISH and the FlowCAM (p=0.16). To increase the accuracy of species specific detection, the FlowCAM was coupled with the oligonucleotide probe (NA1) to target *A. catenella* cells within mixed natural samples. Imaging flow cytometry is an important new technology providing precise and simultaneous measurements of phytoplankton populations to facilitate their continuous monitoring.

**Keywords:** Harmful algal blooms; monitoring; *A. catenella*; imaging flow cytometry; oligonucleotide probe
INTRODUCTION

An automated system capable of identifying and quantifying phytoplankton at species level would be a valuable addition to routine monitoring programs worldwide. This idea of automated monitoring has received growing interest over the last decade (Thyssen et al., 2008; Benfield et al., 2007; Embleton et al., 2003). Traditional cell quantification methods using microscopy techniques can be both tedious and time consuming and rely on a ‘trained eye’ to identify specific species. The precision of enumeration by microscopy is also thought to decrease with cell density (Lund et al., 1958), potentially compromising the early detection of harmful algal species in coastal systems. The FlowCAM, developed by Fluid Imaging Technologies provides an automated rapid procedure incorporating flow cytometry, digital imaging and microscopy, allowing for the real-time counting and sizing of particles (Sieracki et al., 1998). Particles are detected either by laser light scatter or fluorescence signaling in a fluid stream controlled by a peristaltic pump. Within one minute >1,000 cells can be measured and quantified, resulting in a higher yield of data and increased statistical significance compared to more traditional methods of monitoring. Previous studies examining the use of the FlowCAM for microalgae analysis found it to be a precise and practical method for both cell enumeration (Buskey and Hyatt 2006; Wang et al., 2010; See et al., 2005) and size spectrum analysis (Alvarez et al., 2011).

The FlowCAM’s ability to capture and store archival images of individual cells is perhaps one of its most advantageous features. Data processed during a sample run can be filed and analyzed at a later stage with no limiting expiry, allowing the assessment of long-term changes within the microalgae community. The FlowCAM can also be customized to quantify cells of a particular morphological structure using pattern recognition algorithms. This imaging software allows the creation of ‘libraries’, designed to enumerate specific species within heterogeneous samples using images of that target species. The FlowCAM instrument is transportable, involves little set up and is run at a low cost. Other automated systems such as the flow cytometer have been successfully developed to count and sort marine microalgae (Collier et al., 2000), but its upper size...
limit is a major inhibiting factor when identifying particles from a mixed field sample (Peeters et al., 1989). As the FlowCAM doesn’t require sheath fluid it can count particles up to 2000 µm in size. The video plankton recorder (VPR) (Davis et al., 1992) is also a valuable automated instrument successfully used to calculate in-situ plankton abundance by combining microscopy with real-time imagery, but it has a lower size limit of ~200 µm. These upper and lower size limits can result in a particular size range of the phytoplankton group being left unaccounted for when it comes to automated methods of identification and enumeration.

Species of the dinoflagellate genus *Alexandrium* are typically held responsible for paralytic shellfish poisoning (PSP) along the North American coastline (Lewitus et al., 2012). The lethal neurotoxin producer *Alexandrium catenella* (Whedon et Kofoid) Balech, 1985 is known to contaminate shellfish and higher trophic levels including finfish and rock crabs within the Monterey Bay area (Jester et al., 2009). With 511 cases of Paralytic Shellfish Poisoning and 32 human deaths recorded over a sixty year period in the state of California (Price et al., 2001), a reliable and consistent method of monitoring is required in the Monterey Bay area; home to a rich diversity of flora and fauna. A submarine canyon which cuts through the Monterey Bay area and at its deepest descends over 2 miles, provides the surrounding shelf region with deep, nutrient-rich waters under the influence of the California current system (Collins et al., 2003). This process impinges on the surrounding ecosystem. For example, the supply of cold, nutrient replete waters provide a habitat which is suitable for species such as *A. catenella*, generally considered a cold-water organism. Although this species constitutes a relatively minor component of the Californian coastal phytoplankton community (Lewitus et al., 2012), *A. catenella* is present in the waters of Monterey Bay all year round (Jester et al., 2009) and requires consistent monitoring.

Here we investigate the possibility of developing the use of the FlowCAM as one of the main methods for analyzing natural phytoplankton samples. We determine its ability to detect the presence of harmful species within mixed communities, particularly low-abundance organisms such as *A. catenella* that might otherwise be missed by
conventional detection methods. Many studies utilizing the FlowCAM for quantitative analysis use its real-time fluorescence application or light scatter detection mode (e.g. Alvarez et al., 2012; Wang et al., 2010; Buskey and Hyatt 2006), and rely on the FlowCAM’s imaging software for species identification. By coupling the FlowCAM with an oligonucleotide molecular probe we increase the accuracy of its species-specific detection. We compare the accuracy and reliability of the FlowCAM with and without oligonucleotide probe application against the current and more traditional methods of detection employed in the California Department of Public Health (CDPH) monitoring program.

**METHODS**

Field samples were collected weekly from Santa Cruz Municipal Wharf (36° 57.48’ N, 122° 1.02’ W) over an eight week period from December 2012 to February 2013. Water samples from three depths of 0, 5 and 10 feet were retrieved using a Niskin bottle and mixed to form an integrated whole water sample. A 5x 10 ft net tow sample was also obtained. All samples were brought to the lab for immediate analysis. Due to the absence of *A. catenella* in the water column during the first four weeks of sampling, both net and whole water samples were spiked with an *A. catenella* culture (ACES isolated from Elkhorn Slough, CA) in log phase growth to concentrations of 100 cells mL⁻¹ and 10 cells mL⁻¹ respectively. Such concentrations are somewhat comparable to the density of *A. catenella* reached in the Santa Cruz wharf during bloom periods. The mean cell concentration during peak bloom periods from 2005 to 2011 was 39 cells mL⁻¹. During the last four weeks of sampling, *A. catenella* was detected in low concentrations within the water column (~2 cells mL⁻¹) with a coincident increase in paralytic shellfish toxins (PSTs) from sentinel mussels, analyzed by the CDPH. In response to this, both spiked and neat (unspiked) water samples were analysed during this period to compare the efficiency of the chosen methods in monitoring blooms of both relatively high and low cell densities. For the quantitative analysis of *A. catenella*, weekly spiked and neat whole water samples were analyzed in triplicate using four comparable methods of detection;
the Utermöhl method, FISH, ‘live’ FlowCAM analysis and ‘probed’ FlowCAM analysis. A two tailed t-test was used to test the variance between cell enumeration results using FlowCAM methods and the Utermöhl and FISH methods.

Firstly, using the traditional Utermöhl method (Reid 1983), a 50 mL sample was preserved with Lugol’s and left for 24 hours in a sedimentation chamber before cells were enumerated using inverted microscopy (Zeiss Axiovert 200). ‘Live’ whole water samples (3 mL) were analyzed on the FlowCAM using the fluorescence trigger mode. A glass flow cell with dimensions 3 mm x 0.3 mm and a 4x objective was the primary set up for FlowCAM use during the survey. *A. catenella* cells were detected and counted using image libraries (with and without a 5% tolerance) previously created with FlowCAM software using natural samples. The tolerance setting allows for a designated margin of uncertainty when using libraries to identify cells. The reliability of the library application was tested by manually counting *A. catenella* cells within each image data set created by the sample run.

For more sensitive detection of *A. catenella* cells within the whole water samples, a probe specific to the North American ribotype (NA1; Anderson et al. 1999, Scholin et al. 1994) of the genus *Alexandrium* was attached to the samples and observed both by epifluorescence microscopy and automated imaging on the FlowCAM. As *A. catenella* is the only known *Alexandrium* species in the Monterey Bay area, this probe presented adequate specificity. Replicate 50 mL samples were filter concentrated onto 1.2 µm polycarbonate filters and preserved. For epifluorescence detection, the filters were preserved in saline ethanol for a minimum of 1hr before hybridization. The probe was hybridized to the sample using the standard fluorescent in-situ hybridization (FISH) protocol adapted from (Miller and Scholin 1998). For use on the FlowCAM, filters were preserved in a saline methanol solution for at least 24 hrs to eliminate all natural pigmentation. Samples were then hybridized using the FISH protocol and re-suspended in 5X SET wash buffer to a volume of ~3 mL. The NA1 probe was labeled with DY547 (non-licensed CY3 equivalent, Oligos Etc., Inc.) dye for detection by the FlowCAM upon exceeding a user-determined threshold level. The gain and threshold context settings on the FlowCAM need to be set in order to specifically complement discrete sample types.
To measure the efficiency of the sample hybridization, the universal negative control UNI-R probe and the universal positive control UNI-C probe were hybridized to replicate samples alongside the use of the NA1 probe for both epifluorescence microscopy (Zeiss AxioImager.A1) and FlowCAM detection. A sample containing no probe was also prepared to check the level of background fluorescence on filters prepared for epifluorescence observation. Levels of cross reactivity of the NA1 probe were investigated prior to the analysis. This was achieved by mixing an aliquot of an *A. catenella* culture with a similar volume of a *Prorocentrum micans* culture (MB1206B, isolated from Monterey Bay, CA) and fixing, hybridizing and analyzing the sample as mentioned above.

A method adapted from Hawkins (2010) was investigated with the purpose of extracting cells from neat water samples using density gradient centrifugation with sodium polytungstate (Sigma 71913). This was tested as a potential method for concentrating down whole water samples to increase accuracy during FlowCAM analysis. The method also eliminates debris from net tow samples, thus reducing the occurrence of false positives. 50 mL of an *Alexandrium* culture (10,000 cells mL\(^{-1}\)) was centrifuged to form a pellet at 300 x g for 15 minutes. The pellet was re-suspended in un-filtered seawater and overlaid onto 5 mL of sodium polytungstate in a 15 mL BD Falcon \(^{\text{tm}}\) centrifuge tube. The sample was centrifuged at 300 x g for 30 minutes. A band of cells aggregated above the sodium polytungstate, while a pellet of debris formed in the bottom of the tube. The band of cells was removed to a 15 mL tube, washed with 0.2 µm filtered seawater (FSW) and centrifuged once again at 300 x g to create a pellet. This pellet was diluted with FSW to a concentration suitable for analysis on the FlowCAM.

Weekly net samples were prepared for a relative abundance study and a side by side comparison of traditional microscopy using a dissecting microscope (Leica MZ125) and automated imagery on the FlowCAM. For relative abundance analysis, an aliquot of net tow was left to settle in a glass Petri dish and analyzed using the Leica dissecting microscope. The relative abundance codes (rare (<1%), present (1-9%), common (10-49%) and abundant (>50%)) were used to calculate the relative percentages of individual species, following the standard protocols for the CDPH monitoring program and as
described in Jester et al., 2009. A relative abundance index was then used to determine a ratio of dinoflagellates: diatoms. To assess the FlowCAM’s ability for estimating relative abundance in live net tow samples, a live 1 mL sample of net tow was run on the FlowCAM using a 4x objective in fluorescence trigger mode. Estimates of the proportion of dinoflagellates and diatoms within the natural sample were calculated using aspect ratio plots and by sorting images in equivalent spherical diameter (ESD). *A. catenella* cells were enumerated using a pre-created image library of the species. The accuracy of the library was tested by manually counting the *A. catenella* cells within the image data. Triplicate counts of *A. catenella* were averaged and presented as a relative percentage of the total diatom and dinoflagellate population. The NA1 probe was also hybridized to 1mL net samples preserved weekly for species specific qualitative detection on the FlowCAM using the same technique as described for the whole water samples. This was done simply to investigate whether the probed cells had sufficient signal to be detected within a heterogeneous sample of increased concentration.

In addition to weekly field samples, an *A. catenella* culture of known density was used to test and compare the accuracy of the various methods of cell identification and enumeration. Each technique was tested using triplicate samples and the average then used as the comparison. 1 mL aliquots of the *Alexandrium* culture were firstly counted using a Sedgewick-Rafter cell. The FlowCAM was used to count 1 mL samples of live culture in fluorescence trigger mode, in addition to 1 mL samples fixed with both formalin and Lugol’s using light scatter mode. 1 mL aliquots of the culture were also hybridized with the NA1 probe as described above and analyzed both by epifluorescence microscopy and using the FlowCAM.
RESULTS

The enumeration of *A. catenella* cells in weekly whole water samples (spiked to 10 cells mL\(^{-1}\)) using various methods of quantification is illustrated in figure 1. There is good correspondence between the FlowCAM ‘live’ sample results and the results from the more commonly employed FISH and Utermöhl methods. Due to the small sample volume (3 mL) used in FlowCAM analysis, it failed to detect the slight increase of *A. catenella* cells during the final four weeks of sampling, when the species began appearing in the water column averaging at 2 cells mL\(^{-1}\). The Utermöhl method best represents this slight increment in cell numbers during the final four weeks of sampling and appears to be the most precise when it comes to cell enumeration, yet it is also the most time consuming. Increasing the volume of sample analysed using the FlowCAM would potentially increase accuracy, but this causes practical issues such as clogging of the flow cell and an increased number of false positives.

NA1 probed samples analysed using the FlowCAM successfully detected the probed *A. catenella* cells within heterogeneous samples. Although it qualitatively detected *A. catenella*, the results were substantially lower compared to other techniques utilized for *A. catenella* cell enumeration, questioning its quantitative ability (Figure 1). Epifluorescence microscopy was used to analyze the polycarbonate filters after the re-suspension of the material for FlowCAM analysis and found ~40% of *A. catenella* cells remaining on the filter. A count produced by the FlowCAM for an *A. catenella* probed
sample was then compared to an epifluorescence microscopy count of the same sample outflow and a similar result was found between the two. This demonstrates the lower counts were due to an issue during sample processing rather than inherent bias in the FlowCAM.

Table 1 shows the weekly mean (of triplicate) *A. catenella* cell counts mL⁻¹ using the FlowCAM ‘live’ method, FISH method and Utermöhl method. A significant difference was found between the cell enumeration results derived from using the FlowCAM system and the Utermöhl method; \( P = 0.01 \), while no significant difference was found between the FlowCAM and FISH method; \( p = 0.16 \). This highlights the potential of automated methods such as the FlowCAM to detect blooms of a relatively low density within mixed natural samples. The Utermöhl method provided a mean cell count closest to the target density of 10 cells mL⁻¹.

Table 1. Weekly *A. catenella* counts (mL⁻¹) using the FlowCAM, FISH and Utermöhl methods. Standard error values are presented for each method. Mean and standard deviation results also expressed.

<table>
<thead>
<tr>
<th>Date</th>
<th>FlowCAM cells mL⁻¹</th>
<th>Standard error</th>
<th>FISH cells mL⁻¹</th>
<th>Standard error</th>
<th>Utermöhl cells mL⁻¹</th>
<th>Standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-Dec</td>
<td>8</td>
<td>1.15</td>
<td>5</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>6-Jan</td>
<td>5</td>
<td>1.15</td>
<td>8</td>
<td>-</td>
<td>9</td>
<td>0.06</td>
</tr>
<tr>
<td>9-Jan</td>
<td>6</td>
<td>0.67</td>
<td>8</td>
<td>1.99</td>
<td>8</td>
<td>0.38</td>
</tr>
<tr>
<td>16-Jan</td>
<td>9</td>
<td>0.67</td>
<td>6</td>
<td>0.85</td>
<td>9</td>
<td>0.33</td>
</tr>
<tr>
<td>23-Jan</td>
<td>5</td>
<td>0.33</td>
<td>9</td>
<td>0.12</td>
<td>14</td>
<td>-</td>
</tr>
<tr>
<td>30-Jan</td>
<td>4</td>
<td>0.58</td>
<td>8</td>
<td>0.62</td>
<td>11</td>
<td>0.68</td>
</tr>
<tr>
<td>2-Feb</td>
<td>4</td>
<td>0.58</td>
<td>7</td>
<td>0.22</td>
<td>11</td>
<td>0.83</td>
</tr>
<tr>
<td>6-Feb</td>
<td>6</td>
<td>0.58</td>
<td>9</td>
<td>-</td>
<td>13</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Mean 5.88 7.50 10.13
SD 1.81 1.41 2.64

The FlowCAM results shown in table 1 represent those counted manually within the image data following a sample run, not calculated by the image library. Table 2 presents the results of three cell enumeration techniques using the FlowCAM’s imaging software;
cell library counts, cell library counts with a 5% tolerance and manual counts within the image dataset. The pre-created library used for the quantitative analysis of *A. catenella* in spiked samples (10 cells mL\(^{-1}\)) was found to have an average error of 60%, while counts verified manually within the image data had an average error of 40%, highlighting the need for improved imaging software. In all three cases, the percentage error increased for neat sample analysis, indicating a decreasing accuracy with decreasing density. As *A. catenella* is a chain forming species, the use of libraries for its identification also caused issue as the FlowCAM was unable to distinguish chain formations. Chains consisting of more than two cells were rarely observed within samples.

Table 2. *A. catenella* whole water cell counts using the FlowCAM with three different modes of cell identification; *A. catenella* image library, *A. catenella* image library with a 5% tolerance and manual identification. The standard deviation and % error are also presented for each triplicate count.

<table>
<thead>
<tr>
<th>Date</th>
<th>AVG lib</th>
<th>SD</th>
<th>% Error</th>
<th>AVG lib + 5%</th>
<th>SD</th>
<th>% Error</th>
<th>AVG Manual</th>
<th>SD</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-Dec</td>
<td>10</td>
<td>1.5</td>
<td>50</td>
<td>10</td>
<td>1.5</td>
<td>50</td>
<td>8</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>6-Jan</td>
<td>5</td>
<td>5.8</td>
<td>50</td>
<td>5</td>
<td>7</td>
<td>50</td>
<td>4</td>
<td>3.2</td>
<td>20</td>
</tr>
<tr>
<td>9-Jan</td>
<td>7</td>
<td>1.2</td>
<td>33</td>
<td>8</td>
<td>2</td>
<td>20</td>
<td>6</td>
<td>1.7</td>
<td>20</td>
</tr>
<tr>
<td>16-Jan</td>
<td>12</td>
<td>1.2</td>
<td>20</td>
<td>13</td>
<td>.6</td>
<td>33</td>
<td>9</td>
<td>0.6</td>
<td>7</td>
</tr>
<tr>
<td>23-Jan</td>
<td>4</td>
<td>1</td>
<td>56</td>
<td>6</td>
<td>.6</td>
<td>38</td>
<td>4</td>
<td>1.5</td>
<td>20</td>
</tr>
<tr>
<td>30-Jan</td>
<td>2</td>
<td>1.2</td>
<td>76</td>
<td>3</td>
<td>1.2</td>
<td>67</td>
<td>5</td>
<td>0.6</td>
<td>30</td>
</tr>
<tr>
<td>30-Jan (neat)</td>
<td>0</td>
<td>0.6</td>
<td>96</td>
<td>1</td>
<td>0.6</td>
<td>91</td>
<td>1</td>
<td>1.2</td>
<td>70</td>
</tr>
<tr>
<td>2-Feb</td>
<td>3</td>
<td>2.9</td>
<td>71</td>
<td>4</td>
<td>4.2</td>
<td>56</td>
<td>4</td>
<td>2.3</td>
<td>40</td>
</tr>
<tr>
<td>2-Feb (neat)</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>100</td>
<td>1</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td>6-Feb</td>
<td>4</td>
<td>1.6</td>
<td>60</td>
<td>4</td>
<td>2.5</td>
<td>56</td>
<td>6</td>
<td>0.8</td>
<td>20</td>
</tr>
<tr>
<td>6-Feb (neat)</td>
<td>2</td>
<td>0.9</td>
<td>83</td>
<td>3</td>
<td>1.1</td>
<td>73</td>
<td>1</td>
<td>1</td>
<td>80</td>
</tr>
</tbody>
</table>

No cross reactivity was observed either in mixed culture samples or natural field samples containing NA1 probed *A. catenella* cells. Yet, when a passing *A. catenella* cell met the fluorescence threshold, the camera on the FlowCAM would take a raw image of all particles within the field of view including debris. The imaging software on the FlowCAM lacks the ability to distinguish between a cell producing the trigger signal and all other particles within the image, resulting in false positives. Diluting the sample with
FSW helped to reduce but not eliminate this problem. This could be fixed by manually verifying the positive samples, but this re-introduces human observations to an otherwise automated method. The creation of a density barrier may also be a viable method of reducing false positives. Sodium polytungstate was used to create a density gradient in a culture of *Alexandrium* sp. cells, and resulted in the successful concentration and isolation of a band of cells. Although this won’t help reduce the number of false positives of other cells, it may help eliminate the occurrence of debris as false positives. No apparent damage was caused to the cells after the centrifugation and their natural pigmentation appeared unaffected when tested using epifluorescence microscopy 1 hr after processing. This allowed the FlowCAM to detect the cells using fluorescence trigger mode.

Both methods used for determining the relative species abundance in weekly (spiked) net tow samples exhibit comparable results. Figure 2A represents the relative abundance of *A. catenella* cells against the proportion of diatoms to dinoflagellates within weekly samples, as calculated using the FlowCAM. *A. catenella* cells manually counted from the image data were used in the abundance calculation, similar to the whole water analysis. Proportions of diatoms and dinoflagellates were estimated using aspect ratio plots and the ESD of the cells.

As the most common genera of diatoms found around the Santa Cruz area are *Thalassiosira*, *Chaetoceros*, *Skeletonema* and *Pseudo nitzschia*, this method proved successful in the discrimination between the two phytoplankton groups. The most common dinoflagellate genera in this area include *Dinophysis*, *Akashiwa*, *Alexandrium*...
and *Prorocentrum*. The only dinoflagellate spp. which was occasionally misidentified as a diatom, based on its elongated morphological structure, was *Ceratium*. Figure 2B represents the relative abundance results estimated using the dissecting microscope. *A. catenella* observed in the spiked net samples (100 cells mL\(^{-1}\)) by means of microscopy fell within the abundance classification ‘present’ (1-9%) each week. This categorization resulted in a slightly higher percentage of *A. catenella* compared to the discrete values as determined by the FlowCAM. The average percentage of *A. catenella* found in spiked net samples using the FlowCAM was 3%. The FlowCAM successfully detected probed *A. catenella* cells within 1 mL net tow samples, yet false positives occurred more frequently as a result of the high cell density. This altered the specificity of *A. catenella* detection.

Figure 3 shows cell enumeration results of an *A. catenella* culture using five methods of quantification. Each result is compared to *A. catenella* cell density as calculated from a triplicate Sedgewick Rafter count of the same culture sample. FlowCAM results for both ‘live’ samples and formalin preserved samples are comparable to the results of the more accustomed method of FISH and Sedgewick-Rafter cell counts. Aliquots of culture probed with the NA1 probe show a 40% loss of cells when run on the FlowCAM, a loss comparable to whole water samples and more than likely also as a result of inadequate resuspension of the material from the filter. *A. catenella* cells preserved with Lugol’s produced the lowest result when run on the FlowCAM as this preservative was found to deteriorate the light scatter efficiency due to its dark colouration.
DISCUSSION

Based on the results of this study, the FlowCAM exhibits the ability to provide an accurate assessment of the phytoplankton community when compared to microscopy techniques. Each sample run on the FlowCAM provides an image data set of all particles within that sample, from which phytoplankton groups can be distinguished based upon their morphological structure. Unlike microscopy methods, data output from the FlowCAM is non-biased and can be archived for further analysis at a later date. The FlowCAM has also expressed potential to detect specific species within samples using both imaging software and oligonucleotide probe labeling. Although both applications have their limitations, the FlowCAM has nonetheless shown its capabilities as a reliable and automated method of phytoplankton monitoring and its beneficial properties are reviewed further within this discussion.

Imaging flow cytometry has many advantages over traditional monitoring methods, including the ability to quantitatively analyze live samples. This allows samples to be observed immediately and avoids the need for preservation techniques which can potentially damage or alter fragile cells by causing both shrinkage and swelling (Menden-Deuer et al., 2001). Alteration of the morphological structure of cells may also result in the species acquiring a wrong identity by the observer. Preserved cells deteriorate over time, making the possibility of re-analysis at a later date limited to a short time frame. For example, Lugol’s preserved samples rapidly deteriorate when exposed to daylight and require storage in a cool, dark environment, within which their viability is generally restricted to an order of months. Little variance was found between cell counts estimated by the FlowCAM in either fluorescence trigger mode or light scatter mode when cells are preserved in formalin. This presents the option of analyzing samples in either a live or preserved state.

The FlowCAM has demonstrated its potential to be a rapid and practical method for the routine monitoring of potential harmful algal species. As demonstrated in this case, it successfully allows for the detection of the toxic species *A. catenella*. We observed enumeration results obtained by the FlowCAM, to be comparable to the results obtained
by FISH methodology. A study by See et al., (2005) also found the FlowCAM to be a reliable indicator of cell abundance when compared to fluorescence microscopy methods. As the time frame of this study was limited to 8 weeks, more extensive research needs to be carried out on the comparability of the FlowCAM with the more commonly employed methods of phytoplankton monitoring. Although *A. catenella* cell densities estimated using the FlowCAM showed no significant difference when compared to FISH results, a significant difference was found between the cell densities generated by the Utermöhl method and the FlowCAM. This needs to be investigated further with a greater volume and wider range of samples. The FlowCAM has shown its ability to detect species of interest using its image recognition software, yet the images need to be manually verified by the user and this can reintroduce observer bias. We found that a higher concentration of target cells mL$^{-1}$ within heterogeneous samples decreased the percentage error of the cell recognition software. Yet even with a concentration of 10 cells mL$^{-1}$ the standard error remained at least 33%. Buskey and Hyatt (2006) also recognized the need for advancements in the FlowCAM’s imaging software. Depending on the particular threshold setting, they found the FlowCAM’s recognition software could misidentify >70% of non-target cells as target cells within natural samples.

Alvarez et al., (2011) demonstrated that the FlowCAM is a more efficient method when compared to microscopy for carrying out size spectrum analysis. In this study, the Utermöhl method proved to be the most precise method of cell quantification, yet it is advised seawater samples are left to settle for ~24hrs before analysis is carried out. In the monitoring of harmful algal species, sample processing is often required to be carried out within a more efficient time frame. Although it may be time consuming, the Utermöhl was the least expensive method utilized in this study, and one which requires little manipulation of samples prior to analysis. In comparison, FISH methods require at least 4-6 hours of processing, yet analysis is less time consuming than the Utermöhl method. Due to the FlowCAM’s imaging analysis requiring manual checking, the time required for analysis of FISH and FlowCAM samples is comparable. If manual application was eliminated, the processing time for a FlowCAM sample would be ~10 minutes. The initial cost of purchasing a FlowCAM is ~70,000 euro, yet the cost of each sample run is
<1 euro. A fluorescent lamp attachment to enable fluorescent microscopy is perhaps the most expensive purchase to allow for FISH methodologies. Specific oligonucleotide probes can cost in excess of 100 euro, in addition to buffer components, polycarbonate filters and microscope slide materials. Aside from its initial cost the FlowCAM is less expensive than FISH analysis and more efficient than the Utermöhl method.

Although it also showed some limitations, the FlowCAM’s ability to detect and image specifically probed cells within heterogeneous samples is a significant advancement within the field of flow cytometry. An automated system such as this would greatly aid the characterization and enumeration of individual cells within natural samples from the marine environment (Simon et al., 1995). A key development for the success of coupling the FlowCAM with oligonucleotide probes would be to improve the quantitative analysis of the probed cells. A major restricting factor is the FlowCAM’s inability to distinguish which cell in the field of view has produced the trigger signal, resulting in all particles within the same field of view being imaged and counted. This can increase the occurrence of false positives and again, requires the manual verification of cells within the image data. Implementing the density barrier technique to concentrate cells within a sample before analysis could help to minimize the occurrence of false positives as it eliminates a large percentage of debris. It also allows the analysis of higher volumes of whole water, potentially resulting in increased accuracy. Probed *A. catenella* cells were also greatly underestimated due to a loss of material during the re-suspension of the sample from the filter. Adapting the centrifuge based FISH method (Sako et al., 2004) would potentially reduce a loss of material as cells undergo hybridization within a suitable medium and not upon a polycarbonate filter.

The real-time imaging tool is a highly valuable component of the FlowCAM and produces a reliable and complete overview of the phytoplankton community structure, potentially highlighting responses to short-term environmental changes. The archived imagery cataloged by the FlowCAM after each sample run creates an attainable source which permits the efficient analysis of all organisms present at a later date; a valued addition to long term monitoring programs. The quality of cells within a preserved
sample will often start to deteriorate after several months, making re-analysis of the samples more difficult and potentially less reliable. In addition to this, the continual storage of preserved samples in a suitable environment may not always be a possibility during long term monitoring efforts. The semi-automated protocol used by the FlowCAM may help lower potential observer bias introduced in time-series caused by changes in microscopists, as well as in the subjective scoring of relative abundance indices, allowing for the development of consistent plankton records as part of ongoing monitoring efforts.

CONCLUSION
An increasing awareness and concern for HAB events on a worldwide scale calls for a method of phytoplankton cell analysis capable of producing an overview of the microalgae community in the water column within an efficient time frame and with reduced manual labour. Although the FlowCAM as an automated method of monitoring requires further development, it shows its proficiency through its ability to target individual species of interest, provide an efficient analysis of the phytoplankton community structure and produce a valuable digital archive of all imaged particles. All of these properties hold great value within a phytoplankton monitoring system.

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REFERENCES


Chapter II


CHAPTER III

Investigating the role of parasitic infections on dinoflagellate bloom dynamics in Cork harbour

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Robin Raine
PhD supervision and guidance

Gerard Fleming
Project guidance
ABSTRACT

Eukaryotic marine parasites have long been considered as potential agents in the control of harmful algal blooms (HABs). *Amoebophrya* and *Parvilucifera* are two globally widespread parasite genera known to infect a number of dinoflagellate species, many of them toxic, and both have been linked to bloom demise in the past. Laboratory experimentation investigating the role of parasitic infection in culture outweighs the number of studies carried out in the natural environment. In this study we attempt to determine the level of influence parasitic infections exert over the bloom dynamics of four dinoflagellate host species within two natural estuarine systems on the southwest coast of Ireland; Cork Harbour and Kilmacsimon Quay. We intended to focus our attention on populations of the highly toxic species *Alexandrium minutum*. Although species of both *Amoebophrya* and *Parvilucifera* were identified in the water column, no infections were confirmed within *A. minutum* cells. The dinoflagellate species *Akashiwo sanguinea*, *Scrippsiella trochoidea* and *Heterocapsa triquetra* were found to be hosts to *Amoebophrya* sp. The highest prevalence of *Amoebophrya* in both 2011 and 2012 was detected in the *H. triquetra* population at 77% and 55% respectively. A substantial decrease in the population was observed after both periods of increased prevalence, yet it did not appear to inhibit the population’s recovery. DNA analysis confirmed the presence of *Parvilucifera* sp. in Cork Harbour, yet light microscopy methods failed to detect the species infecting any of the dinoflagellate populations. This work constitutes the first report of both *Amoebophryidae* and *Parvilucifera* infections in dinoflagellates populations in Irish coastal waters creating the opportunity for further analysis.

**Keywords**: Harmful algal blooms; parasites; *Amoebophrya*; *Parvilucifera*; prevalence rates
INTRODUCTION

Dinoflagellate blooms of intense cell density can potentially affect the water quality within a coastal embayment or estuarine system, whether they are toxic or non-toxic. Such dense blooms can lower oxygen levels, causing the harm or even death of farmed shellfish and finfish. Shellfish farms are particularly vulnerable to toxic algal species whose toxins accumulate within the bivalve tissues of filter feeding shellfish, causing a range of serious gastrointestinal or neurological illnesses if ingested by humans. Harmful algal blooms (HABs) have also impacted on bird (Jessup et al., 2009), mammal (Van Dolah 2005) and other marine faunal populations (Silke et al., 2005). As regions of coastline continue to be developed for both recreational and cultivation purposes, the worldwide distribution of HABs and their associated environmental and health impacts have become a growing topic of concern. A strategy designed to encourage bloom dissipation is highly desired in enriched coastal environments where HABs are known to recur annually.

A biological method of bloom control was first suggested by Taylor (1968), whereby eukaryotic marine parasites would be introduced into the water column to propagate within dinoflagellate host species, inducing host mortality and eventually bloom demise. The hypothesis was later dismissed by Nishitani et al., (1985) due to the specificity of the potential parasites in the biota being questioned. Since then, molecular tools have allowed for more detailed analysis of species identification and genetic diversity. The syndinian parasite Amoebophrya ceratii (MALV II) was found to be a species complex with the capacity to parasitize over 40 dinoflagellate species, yet have a high degree of host specificity due to several host specific taxa (Coats et al., 1996). Although this obligate parasite is not restricted to dinoflagellate hosts, it is commonly found within coastal dinoflagellate populations (Chambouvet et al., 2008; Alves-de-souza et al., 2012; Mazzillo et al., 2011). Individual dinospores penetrate the host cell and undergo nuclear replication, maturing into a ‘beehive’ shaped trophont within either the nucleic or cytoplasmic material depending on the host species (Kim et al., 2004). After ~48 hrs, a motile vermiform emerges and releases between 60 and 400 swimming dinospores (Coats
et al., 1994; Coats and Park 2002) capable of re-infecting healthy host cells. This high level of offspring production is potentially a contributing factor in the parasite’s ability to prevail and regulate blooms.

The perkinsozoan genus *Parvilucifera* is an intracellular parasite also known to infect dinoflagellate populations, and it has been suggested to it play a role in bloom decline (Gisselson et al., 2002). *P. infectans* was the first discovered species of this genus (Noren et al., 1999). A study by Noren et al. (2000) found *P. infectans* not to be host specific, yet Lepelletier et al. (2013) found dinoflagellate host resistance to an isolated ribotype of *Parvilucifera* to be highly variable among host strains. *Parvilucifera* is known to infect over 17 dinoflagellate host species to date (Park et al., 2004) and is commonly observed infecting *Alexandrium* spp. (Noren et al., 1999; Figueroa et al., 2010; Garcés et al., 2010). The infective process of *Parvilucifera* involves a flagellate zooid invading the host cell and maturing into a spherical dark sporangium while digesting the host cell contents. The sporangium ruptures within ~48 hrs, releasing up to 500 zooids into the water column (Figueroa et al., 2008).

The parasitic flagellates described above are frequently found in coastal and estuarine systems but their level of impact on the algal community is not yet fully established. While the infection prevalence rate of *Parvilucifera* spp. within dinoflagellate host populations is still largely unknown, *Amoebophrya*, the more commonly studied genus, has been noted to reach relatively high prevalence rates of 40-80% within host dinoflagellate populations (Cachon 1964; Coats et al., 1996; Maranda 2001; Chambouvet et al., 2008). Other studies have shown *Amoebophrya* spp. to have relatively low prevalence rates (<9%), and an unlikely impact on dinoflagellate bloom regulation (Gisselson et al., 2002, Salomon et al., 2003, Salomon et al., 2006).

The North Channel, located in Cork Harbour on the south coast of Ireland, is a shallow and retentive estuarine environment. A diverse range of dinoflagellate species are present annually within the estuary, including the neurotoxin producing species *Alexandrium minutum* posing a threat to local aquaculture sites, and the surfactant producing species
Akashiwo sanguinea with the potential of causing harm to local protected bird populations (Jessup et al., 2009). The sessile resting cyst stage of *A. minutum* grants stability to the population which appears annually in the water column, reaching intense bloom status (>500,000 cells L⁻¹) approximately every seven years (Cosgrove et al., 2014). Introducing a host specific marine parasite to act as a biological control in this area would potentially allow the dissipation of nuisance blooms known to cause substantial economical and environmental damage.

In this study we look at the effect of two globally widespread parasites in their natural environment within Cork Harbour and other inlets on the south coast of Ireland, and their influence on the bloom dynamics of four dinoflagellate host species. A wide variety of infection prevalence rates within marine dinoflagellate populations has been reported; we aimed to determine the threshold prevalence percentage which can potentially cause bloom demise in two natural estuarine systems. We also investigate the capability of *Amoebophrya* overwintering in the freshly formed cysts of two potential dinoflagellate hosts from the sediment of the North Channel. This work constitutes the first report of both *Amoebophryidae* and *Parvilucifera* infections on dinoflagellates in Irish coastal waters.

**METHODS**

1. Study area, sample collection and processing: The North Channel
The first study area was located on the south coast of Ireland within Cork Harbour (figure 1A). Sampling was carried out in the North Channel (figure 1B); a sheltered estuary located in the north eastern half of the harbour, approximately 9 km in length and between 0.5 and 1km in width. The channel connects to the main harbour towards its eastern end and it is here that water depths are greatest, averaging 7 m depth at station R. Towards the western end of the channel, water depths average at ~2 m, exposing extensive mudflats on low tide. A tidal excursion of 6-8 km results in water in the western end of the North Channel to be of a retentive nature. During the summer months
of 2011 and 2012, weekly water sampling was carried out along a transect in the North Channel. Five stations (R, Q, P, O, NO) were sampled during the bloom period between mid-May to mid-July (Ní Rathaille 2007) located at intervals of ~0.5 km along the channel. Surface water samples were collected using a bucket, while sub-surface samples were collected using a 5L Niskin bottle. Sediment samples from four locations in the North Channel were also collected during a survey in March of 2011. An Ekman Birge bottom sampler (Hydro Bios, Kiel) was used to manually retrieve sub-tidal sediment samples. Sub-samples of the collected sediment were extracted in triplicate using sawn-off 50 mL syringes. The core was pushed down into the collected sediment sample while holding the plunger static to ensure no air entered the sub-sample. The cores were sealed with Parafilm and stored in the dark at 4 °C until further analysis.

Figure 1. The study area. A Bathymetric map of Cork Harbour, Ireland, highlighting the North Channel located in the northeastern half of the harbour. (B) Map of the North Channel showing the five water sampling stations occupied on each survey. The location of temperature sensor is also indicated.
Environmental sampling
During each sampling survey in the North Channel water temperature and salinity measurements were recorded using a WTW iT90 temperature salinity probe. The measurements were taken at 1m intervals from the surface to the sea bed at each station. A TidbiT data logger (Onset, Maine) permanently attached to a mooring deployed between stations O and N-O (Figure 1B) also recorded hourly water temperature. Secchi disk measurements were taken at each station. Incident photosynthetically active radiation was measured with a Licor LI190 quantum sensor. This was mounted 3 m above ground level in a shade free environment on the northern shore of the North Channel and integrated hourly data were logged with a LI1000 data logger.

Live sample collection and analysis
500 mL surface and sub-surface water samples were collected and filtered through a 5 μm mesh before backwashing into 50 mL tissue culture flasks using 0.22 μm filtered seawater (FSW) for live cell analysis and isolations of live material. All live material was stored close to the observed water temperature (~15 °C) during transportation, and transferred the same day to an incubator set at an irradiance of 100 μE.m\(^{-1}\)sec\(^{-1}\) with a 14:10 light: dark cycle and temperature of 15 °C. Within 24 hours of collection, all live samples were observed using both light and epifluorescence inverted microscopy using an Olympus CKX-41 microscope fitted with a 100W epifluorescence lamp. A U-MWB fluorescence cube with a 450-480 nm excitation filter and a 515 nm barrier filter was used to highlight the natural green fluorescence of *Amoebophrya* cells both in its free-living stage and within the host cell. Infected live host cells were identified, isolated and transferred into aliquots of corresponding culture strains originating from the North Channel. The infected cultures were kept in \(\frac{1}{2}\) IMR medium (As described by Eppley et al., 1967) at 15 °C and under irradiance of 100 μE.m\(^{-1}\)sec\(^{-1}\) with a 14:10 light: dark cycle. Host specificity of *Amoebophrya* was tested by isolating infected host cells of *Akashiwo sanguinea* and *Heterocapsa triquetra*, and cross-infecting 1 mL samples of the non-corresponding host cultures of *A. minutum*, *A. tamarense*, *Prorocentrum micans*, *Scrippsiella trochoidea*, *A. sanguinea* and *H. triquetra* in triplicate (all host strains originated from the North Channel). An aliquot of each host culture was analysed after
48-72 hours to note the presence/absence of infection. Dinoflagellate species were also examined by light microscopy to detect infection by *Parvilucifera* spp based on previous morphological descriptions and illustrations by Garcés *et al.*, (2013).

**Cell enumeration**

50 mL neat water-samples for cell enumeration were stored in tissue culture bottles and preserved with either 0.4 mL Lugol’s iodine or 1 mL 20% buffered formaldehyde solution (0.8% final concentration). Samples were kept at room temperature in the dark until enumeration was carried out using inverted microscopy as described in McDermott and Raine (2010).

**Fluorescent in situ hybridisation**

2L water samples were collected and pre-filtered through a 150 µm mesh sieve for additional cell enumeration and *Amoebophrya* infected host prevalence calculations using fluorescent in situ hybridisation (FISH). The sample was then filtered onto a 47 mm diameter 5 µm nylon mesh filter and backwashed into a 50 mL polypropylene centrifuge tube using GF/F filtered seawater (FSW). All samples for FISH analysis were stored in the dark at 4 °C for up to 6 hours. The samples were then centrifuged to a pellet (4000g, 10 min) and the remaining formalin solution was removed using a gentle vacuum careful not to disturb the pellet. The pellet was then finally resuspended in 14 mL ice-cold methanol and stored at -20 °C until analysis. The FISH method used to hybridise the probes to the samples was based on the method designed by Miller and Scholin (1998). The MinA and TamB oligonucleotide probes (Touzet *et al.*, 2008) were hybridized to the North Channel samples for the enumeration of both *A. minutum* and *A. tamarense* as described in Cosgrove *et al.*, (2014). Using the same method, the general *Amoebophrya* ALV01 probe (5’- GCC TGC CGT GAA CAC TCT -3’) (Chambouvet *et al.*, 2008) was also hybridized to the North Channel and the Kilmacsimon Quay samples collected in both 2011 and 2012 to calculate the prevalence of *Amoebophrya* within specific host species.
Catalysed reporter deposition fluorescent in situ hybridisation
Weekly samples were collected for the quantitative analysis of *Amoebophrya* dinospores using the catalyzed reporter deposition fluorescent in situ hybridisation (CARD-FISH) method. A 50 mL water sample was filtered through a 10 µm mesh and fixed using 5 mL formaldehyde (1% final concentration). The samples were stored in the dark at -20 °C for at least 3-4 hours before being filtered onto 1.2 µm polycarbonate isopore membrane filters using a vacuum pressure <100 Hg. Each filter was then dehydrated using 1 mL of ethanol at 50, 80 and 100% and stored in 1.5 mL Eppendorf tubes at -80 °C. A CARDFISH method adapted from Chambouvet *et al.*, 2008 was utilized for the detection and quantitative analysis of *Amoebophrya* dinospores within samples from both study areas. Once thawed, the filters were placed on a glass slide and 10 µl of 5ng mL⁻¹ horseradish peroxide ALV01 probe mixed with 100 µl of hybridization buffer (0.9 M NaCl, 20 Mm Tris, 0.01% SDS, 10% blocking reagent, 40% formamide) was added. The slide was placed in a humid chamber at 35 °C for at least three hours. The filter was then transferred onto a 2.5 mm filter holder attached to a vacuum manifold via 2-way Leur Lock stop cocks used to filter down excess buffer (see figure 2). Sawn off 50 mL centrifuge tubes were secured to the filter holders to contain the buffer solutions throughout the process. Preheated wash buffer (5 mM EDTA, 0.01% SDS, 20 mM Tris, 0.056 M NaCl) was added to the filter and incubated for 20 minutes at 37 °C. This step was repeated twice. TNT buffer (0.1M Tris, 0.15M NaCl, 0.05% Tween) was added to the filter and incubated at room temperature for 15 minutes. The fluorochrome solution of a 1:20 ratio (1 volume of fluorescein tyramide, for every 20 volumes of dextran sulfate and amplification diluent) was prepared and added to the filter for a 30 minute incubation period in the dark and at room temperature. Pre-heated TNT buffer was added to the filter and incubated at 55 °C for 20 minutes; this step was repeated twice. Distilled water was
added to the filter before and after adding 50 μl of 5 μg mL⁻¹ DAPI. The filter was placed on a glass slide with 50 μl of antifade (Citifluor) and a cover slip was secured over it. All filters were kept in the fridge and analysed within 24 hours.

**DNA extraction and PCR reaction**

A 50 mL water sample taken weekly at each station was preserved in Lugol’s and stored in the dark for DNA extraction. The samples were later filtered onto 25 mm diameter 0.1 μm membrane filters (Millipore) and stored at -80 °C until further analysis. The process of DNA extraction firstly involved cell disruption achieved by bead beating in a ribolyser (Thermohybaid) followed by a spin column format using a DNeasy® plant mini kit (Qiagen). The prepared DNA was quantified using a Nanodrop spectrophotometer. Two *Parvilucifera infectans* specific primers Parvi1-R and Parvi2-R targeting a partial 18s rRNA gene fragment (designed by Johansson *et al.*, 2006) were used to detect the perkinsozoan parasite in the North Channel samples. Each primer was combined with the eukaryotic primer EUK1272F (Janson *et al.*, 2000) for separate PCR reactions. Both reactions were run in triplicate with a negative control (*A. minutum* culture strain CK.p17: the North Channel) and positive control (*P. infectans* culture strain 100-a: Penzé estuary, France). The final volume for the reactions was 50 μl and comprised 1x green Go Taq® buffer, 2 mM MgCl₂, 200 μm of each deoxynucleoside triphosphate (dNTP), 0.1 μm of each primer and 0.25 unit of Go Taq® Polymerase. The amplifications were carried out in a TECHNE TC-5000 thermocycler and involved initial incubation at 94 °C for 8 minutes, followed by 40 amplification cycles of 30s at 94 °C, 1 min at 50 °C, 2 min at 72 °C and a final incubation for 10 min at 72 °C. DNA fragments were visualized under UV illumination on a 1% agarose 1X TBE (Tris-boarate-EDTA) buffer gel stained with Syber Safe® (Invitrogen). Four samples from 2011 and two from 2012 which produced an amplification signal of ~300bp were purified using a Nucleo-spin gel and PCR clean up kit (Macherey –Nagel) and sequenced with the Parvi1-R primer (GATC-Biotech). The sequencing results were aligned using GeneDoc.
Cyst isolations
For cyst quantification, the top 1 cm of sediment from each core was homogenized, weighed and transferred to a 100 mL glass Schott bottle, to which 40 mL of FSW was added before ultrasonicating for 3 minutes at 23kHz. The sample was then sieved through an 80 µm sieve and collected onto a 20 µm sieve before being backwashed into a 15 mL centrifuge tube with FSW. The cysts were extracted using density gradient centrifugation with sodium polytungstate (see Bolch 1997). Both *Scripsiella sp.* and *A. minutum* cysts were enumerated using a Sedgewick-Rafter cell at x100 magnification using an Olympus CK-41 microscope. Sediment from four cores found to contain the highest cyst density, all originating from the western end of the North Channel, were then prepared and sieved as above, excluding the sonication and centrifuging steps so as not to cause damage to the cysts. To eliminate some of the debris, the sample was placed in a small beaker (25 mL) and swirled gently by hand to gather the heavier material at the bottom and leave the cysts in suspension. The suspended material was transferred to a 3 mL glass chamber and cyst isolations were carried out using a glass micropipette. The isolated cysts were washed once in FSW before being transferred individually to wells of a 96 well plate. A total of 96 cysts of both *A. minutum* and *Scripsiella sp.* cysts were collected. All cysts were examined under epifluorescence microscopy for the natural green fluorescence of *Amoebophrya* using the filter set described previously, and subsequently stored at 15 °C under irradiance levels of 100 μE.m⁻¹.sec⁻¹ with a 14:10 light: dark cycle. During the following month, the cysts were observed twice a week for signs of successful germination and/or fluorescing pigments.

2. Study area, sample collection and processing; Kilmacsimon Quay
A second study site known as Kilmacsimon quay (figure 3), located along the lower Bandon estuary in Co. Cork was also sampled. Located ~30km from the North Channel sampling site, this area was chosen due to its annual intense (10⁶ cells L⁻¹) blooms of *Heterocapsa sp.* Weekly samples were collected as near to high tide as possible due to substantial tidal reach and variance in the salinity between tides. Surface water samples were collected weekly using a bucket lowered from the pier. Six weeks of sampling in 2011 and seven weeks in 2012 were carried out during the summer period of May to July.
Live samples in addition to those preserved for cell enumeration and FISH were collected and stored as described above, while CARDFISH samples (processed as above) were also collected during both years. Surface temperature and salinity measurements were recorded weekly from the pier, along with turbidity using a secchi disk.

![Figure 3. Map showing Kilmacsimon Quay study area on the river Brandon. Inset showing location of Kilmacsimon Quay in Ireland.](image)

**RESULTS**

**The North Channel**

**Temperature, salinity and irradiance**

The mean daily temperature in the North Channel for 2011 and 2012 is shown in figure 4, while the mean daily underwater irradiance (PAR) averaged for 2011 and 2012 is illustrated in figure 5. Increased dinoflagellate spp. abundance is generally observed during the months of June and July in the North Channel when temperatures reach 15 °C.
and the integrated water column average irradiance exceeds 100 $\mu$E·m$^{-2}·$s$^{-1}$. Over the eight week sampling period in 2011, the temperature and salinity measurements taken at surface across the channel averaged at 15.6 °C and 29.9 PSU respectively. While in 2012 the surface temperature averaged at 14.95 °C and salinity at 27.4 PSU. Figure 6 shows weekly salinity and temperature readings for 2011 and 2012, averaged across the North Channel. Lower salinity measurements recorded during sampling periods in 2012, predominantly week 5 and 7, followed periods of increased rainfall. June 2012 was reported as having the most rainfall since records began in 1981, with rainfall data (mm) from Co. Cork showing a 280% increase on the average rainfall in June. Following periods of intense rainfall, the salinity values at stations NO and R are often lowered by the land runoff and the Ballynacorra River respectfully. This may account for the higher level of variation between stations in 2012. Secchi depth values ranged from between 1.1 m and 1.4 m in both 2011 and 2012. In 2012, the highest recorded dinoflagellate cell biomass of 80,000 cells L$^{-1}$ was observed during a neap tide on June 27th. It followed the lowest spring tide in June, around the time of the summer solstice. This resulted in reduced tidal flushing and allowed time for bloom development, which is thought to require at least 10 days. During the summer solstice period, the ocean's tides are at their lowest ebb of the year.
Live sample analysis

Both *Amoebophrya* free-living dinospores and maturing trophonts within host cells were observed using epifluorescence microscopy within the live samples collected in the North Channel. It was apparent that *Heterocapsa triquetra*, *Akashiwo sanguinea* and *Scrippsiella trochoidea* were the only dinoflagellate species acting as hosts to the syndinian parasite in both 2011 and 2012, with *H. triquetra* being the most frequently observed infected species. No infected cells of *A. minutum* were observed during either year. Cells of *A. sanguinea* and *H. triquetra* infected by *Amoebophrya* were successfully established in culture in 2012 by isolating a single infected cell of each species into the corresponding host strain culture which originated from the North Channel. It is suggested the *Amoebophrya* species found in the North Channel were found to be host specific after the addition of *H. triquetra* and *A. sanguinea* infected host cells, in a late stage of maturation, into various dinoflagellate culture strains from the North Channel resulted in 0% prevalence of the parasite (see Table 1). *Parvilucifera* infections failed to be observed within any of the dinoflagellate populations during light microscopy analysis of the live samples.
Table 1. Cross infection results indicating a prevalence of infection only within corresponding host cultures.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dinoflagellate sp.</th>
<th>Infected A. sanguinea</th>
<th>Infected H. triqueta</th>
</tr>
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<tbody>
<tr>
<td>CHP.03</td>
<td>A. tamarense</td>
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<td>-</td>
</tr>
<tr>
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<td>A. minutum</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>CH9902</td>
<td>H. triqueta</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Cell enumeration

Weekly cell densities of the *Amoebophrya* host species *H. triqueta*, *S. trochoidea* and *A. sanguinea* during the summer period of 2011 are presented in figure 7A. Out of the three hosts, *H. triqueta* had the highest recorded cell density of 40,000 cells L$^{-1}$ during both week 1 (May 25$^{th}$) and 3 (June 9$^{th}$) of sampling. Both periods of increased cell density did not last more than seven days, and by week 4 (June 16$^{th}$) the bloom had collapsed with cell densities remaining at <3,000 cells L$^{-1}$ for the rest of the summer period. *A. sanguinea* and *S. trochoidea* cell densities didn’t rise above a magnitude of $10^2$ and $10^3$ cells L$^{-1}$ respectively throughout the sampling period. The highest record of *S. trochoidea* cell densities ($> 8,000$ cells L$^{-1}$) was observed during week eight (July 13$^{th}$), a time when the lowest cell densities of both *H. triqueta* and *A. sanguinea* were observed.

Weekly cell densities of *H. triqueta*, *A. sanguinea* and *S. trochoidea* were also recorded in 2012 (figure 7B). The bloom dynamics of *H. triqueta* and *A. sanguinea* were of a similar pattern, showing cell density increases during the second week of sampling and again during week 6, yet not exceeding 8500 cells L$^{-1}$ and 6700 cells L$^{-1}$ respectively. *S. trochoidea* showed slight increases in cell density during week 3 and week 6. All three host populations experienced a bloom collapse during week 7. Yet each population managed to recover, and maximum recorded cell densities of 8,500 cells L$^{-1}$ and 29,000 cells L$^{-1}$ were observed for *H. triqueta* and *S. trochoidea* respectively during week 8.
Amoebophrya prevalence

Free-living *Amoebophrya* dinospore cells and infected host cells were successfully detected and enumerated within natural samples using CARD-FISH and FISH methods respectively. A free living *Amoebophrya* dinospore cell of 5-7 µm and naturally fluorescing under epifluorescence microscopy is illustrated in figure 8. A Calcofluor stained infected *H. triquetra* host cell is illustrated in figure 9A, while figure 9B shows the same cell naturally fluorescing. The typical beehive structure of a mature *Amoebophrya* trophont is illustrated in figures 9A and 9B.
Weekly *Amoebophrya* dinospore densities are plotted with the host cell densities of *H. triquetra*, *S. trochoidea* and *A. sanguinea*, measured in cells L\(^{-1}\) in figures 10A-C respectively. In 2011, *H. triquetra* cells were found to have the highest correlation with dinospores cells (correlation coefficient of 0.8), as both relative densities followed a similar pattern for the first four weeks. Infection prevalence rates of *H. triquetra* are also shown in figure 10A. During week two, the prevalence of *Amoebophrya* in *H. triquetra* (% of infected cells) was low at 1.6%. It increased to 25% by week 3, yet the *H. triquetra* population appeared to be unaffected as the observed cell density was an order of magnitude higher than the previous week. By week 4, the infection prevailed at 77%. This appears to have impacted on the bloom as cell densities fell to <800 cells L\(^{-1}\) and failed to recover above 2,000 cells L\(^{-1}\) for the rest of the sampling period. A low correlation coefficient of 0.3 was found between *A. sanguinea* and *Amoebophrya* cell densities. However, *A. sanguinea* cell densities remained <1000 cells L\(^{-1}\) during the sampling period. *A. sanguinea* cell densities recovered after a sudden decline in week four, yet did not exceed 1,000 cells L\(^{-1}\) throughout the eight week sampling period. No
correlation was found between dinospore and \( S. \ trochoidea \) cell densities and although infections were observed within live samples, it appeared to have had no effect on the magnitude of the bloom. It remains unknown as to why dinospore cell densities reached >11,000 cells L\(^{-1}\) during week 6. Calcofluor analysis found \( H. \ triquetra \) cells to be the most commonly infected host during week 6 with a prevalence of 48%, yet the previous week prevalence was 57% when dinospore density remained at 1,100 cells L\(^{-1}\). It is therefore unlikely \( H. \ triquetra \) infections contributed to this rise in dinospore density.

Weekly \( Amoebophrya \) dinospore densities plotted with \( H. \ triquetra \), \( S. \ trochoidea \) and \( A. \ sanguinea \) cell densities is shown for 2012 in figures 11A-C respectively, with infection prevalence rates of \( H. \ triquetra \) also illustrated in figure 11A. A correlation coefficient of 0.7 was found between \( H. \ triquetra \) and \( Amoebophrya \) dinospore cell densities. A 10% infection prevalence rate observed during week 6 did not appear to impact on the \( H. \ triquetra \) population; it was only when the prevalence rate the following week rose to 55% that a sudden decline in the cell density was

![Figure 11. Weekly average \( Amoebophrya \) dinospore densities cells L\(^{-1}\) measured from stations NO-R in the North Channel, plotted against weekly average \( H. \ triquetra \) (A), \( S. \ trochoidea \)(B) and \( A. \ sanguinea \)(C) cells L\(^{-1}\) in 2012. The prevalence rate represents the proportion of \( H. \ triquetra \) cells infected with \( Amoebophrya \) weekly.](image-url)
observed. The bloom had recovered by week 8, peaking at 8500 cells L\(^{-1}\), appearing unaffected by an infection prevalence rate of 13%. A correlation coefficient of 0.5 was found between both *A. sanguinea* and *S. trochoidea* weekly cells densities and the *Amoebophrya* dinospore cell densities. *A. sanguinea* was the most commonly infected host during week 3 and 4, and so can be suggested as the most likely host responsible for the increase in dinospore cell densities to 30,000 cells L\(^{-1}\) in week 4. All three hosts may have contributed to the dinospore peak of 77,000 cells L\(^{-1}\) during week 6, as a decline in each population was observed the following week. If the increased dinospore density was responsible for host cell decline, it only managed to temporarily suppress each population. The highest rate of infection in the *H. triquetra* population in both 2011 and 2012 was observed one week after the highest recording of *Amoebophrya* dinospore cells L\(^{-1}\) in the water, indicating a link between the parasite and host population dynamics.

Cyst isolation analysis
Examination of the freshly isolated *A. minutum* and *S. trochoidea* cysts using light and epifluorescence microscopy showed that all cells appeared healthy and that there was no sign of green fluorescing pigments. Chlorophyll was the only pigment observed, fluorescing red. Within four weeks, >80% of both species had germinated. The vegetative cells were also examined for green fluorescence but no infections were detected. After several rounds of cellular division, the *S. trochoidea* cells formed what appeared to be temporary cysts in four of the wells. These temporary cysts had an unhealthy appearance (no red accumulation visible within the cyst), and when examined under epifluorescence microscopy they expressed a suspicious green hue. Fresh FSW was added to each of the four wells and they were incubated in the conditions outlined above, but within three weeks the green fluorescence had faded and the cysts appeared to be dead. It is unlikely that the green coloration was linked to parasitic activity, as the cells had successfully germinated and divided.

Detection and genome sequencing of *Parvilucifera* sp.
No *Parvilucifera* spp. infections were observed in live samples from either 2011 or 2012. Using weekly preserved samples, an attempt was made to detect the genus based on its
Chapter III

The Parvilucifera sp. was identified using PCR techniques with the P. infectans specific primer Parvi-1 and P. infectans culture control. It was detected in 2011 during the last four weeks of sampling and its highest concentration was found during week 7 (July 6th). Using the same primer, PCR also detected the parasitic genus in the 2012 samples. It first appeared during week 3 and 4 and then again during weeks 6 and 7. Although Parvilucifera did not appear to be infecting A. minutum cells when observed by microscopy, increased DNA concentrations of the parasite occurred during times of increased A. minutum cell concentrations in both years (figure 12A, B). Shortly after the appearance of Parvilucifera in the water column a collapse of the A. minutum bloom was observed. This occurred in both 2011 and 2012. A BLAST search confirmed the identity of Parvilucifera sp. (~200bp) in the North Channel and it was found to share 100% identity with a strain of P. sinerae isolated from Arenys de Mar Harbor in Mediterranean waters (EU502912; Figueroa at al., 2008), 99% identity with five strains of P. infectans clones originating from Swedish waters (DQ113422, DQ113423, DQ113425, DQ113426 and DQ113427; Johansson at al., 2006) and one P. infectans species isolated from Swedish waters (AF133909; Johansson at al., 2006). Figure 13 shows the phylogenetic analysis of the sequence alignment of all strains. No difference was found (216 bp compared) between the Parvilucifera strain from the North Channel, P. sinerae from the Mediterranean and one of the P. infectans clones from Swedish waters (DQ113423), although two mutation deletions were present in the clone sequence. Two differences in base pairs were noted.

Figure 12. Weekly maximum A. minutum cells L^{-1} recorded in the North Channel. Shaded areas indicate the detection of Parvilucifera in the water column.
between our *Parvilucifera* sp. and the other four clone strains of *P. infectans* and one isolated species from Swedish waters.

Table 2. Species designations and GenBank accession numbers relative to the rDNA sequence alignment in figure 13. CH refers to Cork Harbour *Parvilucifera* sp.

<table>
<thead>
<tr>
<th>Code designation</th>
<th>Species</th>
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<tr>
<td>gi 70959253</td>
<td><em>P. infectans</em> (clone)</td>
<td>DQ113423</td>
</tr>
</tbody>
</table>

Figure 13. The Genedoc sequence alignment of the 18s r DNA gene fragment amplified for the identification of *Parvilucifera* sp. detected in the North Channel, Cork Harbour.
Kilmacsimon Quay

Environmental data
In 2011, the average temperature and salinity recorded at Kilmacsimon quay during high tide was 16.5 °C and 10.5 PSU. The salinity was greatly influenced by tidal effects and dropped as low as 3 PSU during low tide. In 2012, an average salinity of 7 PSU and temperature of 16 °C was recorded, with an average turbidity of 1m. Table 3 shows weekly salinity (PSU) and temperature (°C) data for the years 2011 and 2012 respectively. In general, lower salinity levels are evident in weeks with a greater time period between high tide and sample collection. Missing data on the 19th of June 2012 was due to a technical difficulty with the salinity probe.

Table 3. Kilmacsimon Quay environmental data during sampling periods in 2011 and 2012. Times of sample collection and times of the nearest high tide are shown along with salinity and temperature levels measured from the pier.

<table>
<thead>
<tr>
<th>Date</th>
<th>Sampling time</th>
<th>High tide</th>
<th>Salinity (PSU)</th>
<th>Temperature (°C)</th>
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<td>14.5</td>
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<td>18:00</td>
<td>7.9</td>
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Host-parasite dynamics

Live analysis of samples collected from Kilmacsimon quay showed *H. triquetra* to be infected by *Amoebophrya* sp. in both 2011 and 2012. No other dinoflagellate sp. was observed in the samples. Figure 14A shows *H. triquetra* cell densities recorded at Kilmacsimon quay in 2011 against the rate of *Amoebophrya* infection prevalence, which didn’t rise above 3% during seven weeks of sampling. Although *H. triquetra* cell densities dropped from $1 \times 10^6$ cells L$^{-1}$ during week one to ~600,000 cells L$^{-1}$ in week two, it doesn’t appear to be as a result of parasitic infection by *Amoebophrya*, as prevalence at this time was only 1.4%. The population had recovered to a density of $3 \times 10^6$ cells L$^{-1}$ by week three. In 2012 (figure 14B), dinospore cell densities decreased with *H. triquetra* cell densities, and no *H. triquetra* cells were recorded in the samples after week four. As in 2011, the *Amoebophrya* parasitic infection does not appear to have been responsible for the bloom collapse as prevalence didn’t exceed 1% during the eight week period.
DISCUSSION

This study investigated the parasitic infections of dinoflagellate populations in the North Channel of Cork Harbour by the marine parasite genera *Amoebophrya* and *Parvilucifera*. The results found the dinoflagellate species *A. sanguinea*, *S. trochoidea* and *H. triquetra* to be infected by *Amoebophrya*. And due to the timing of increased *P. infectans* concentrations in the water column during peak *A. minutum* bloom periods, there is a potential the *A. minutum* population was playing host to this parasitic species. Although none of the three species infected by *Amoebophrya* are toxin producers, *A. sanguinea* is a producer of water soluble surfactants and regarded as harmful to the surrounding environment. Both *H. triquetra* and *S. trochoidea* can also be regarded as nuisance species when they bloom in high densities. The highest prevalence rates of *Amoebophrya* in the North Channel in both 2011 and 2012 were detected in the *H. triquetra* population, when infection rates reached 77% and 55% respectively. Both periods of increased prevalence were observed one week after maximum *Amoebophrya* dinospore and increased *H. triquetra* cell densities were recorded. It is suggested these high infection rates impacted on the host population as within 7 days *H. triquetra* cell densities had reduced by two orders of magnitude. In 2011, prevalence rates of >50% continued to suppress the population during the remainder of the sampling period, while in 2012, the parasitic infection acted only as a temporary suppressant, as *H. triquetra* cell densities peaked one week later. This indicates *Amoebophrya* may be capable of suppressing a bloom of *H. triquetra*, yet only temporarily as it fails to prevent the population recovering from an epidemic infection; Coats *et al.* (1996) determined a parasitic infection which exceeded 20% to be an ‘epidemic infection outbreak’. This questions the concept of introducing marine parasites into an ecosystem to act as a biological control during HAB events.

*Amoebophrya* sp. was also identified within *H. triquetra* cells in the estuarine system located at Kilmacsimon Quay, where a similar infection prevalence rate to the North Channel was expected to be observed in the population. Although the estuary is highly influenced by the tide, it was presumed cell densities of up to $1.1 \times 10^7 \text{ L}^{-1}$ would allow
for increased spread of infection based on increased cell contact, but infection rates did not exceed 3% during either 2011 or 2012. Bloom densities of the order of magnitude of $10^6$ and $10^7$ cells L$^{-1}$ were found to correspond with salinity levels of $>13$ PSU, while cell densities didn't exceed 500,000 cells L$^{-1}$ when salinity levels were below 10 PSU. Tidal flushing appears to be an influential factor on *H. triquetra* bloom magnitude in the lower Bandon estuary at Kilmacsimon Quay, and possibly the reason why the *Amoebophrya* infection rate remained low. A similar study by Chambouvet *et al.*, (2008) carried out in the Penzé estuary, a macro-tidal area with a large tidal reach, found *Amoebophrya* to be infecting over 30% of the *H. triquetra* population. This indicates the parasite has the ability to succeed in open estuarine systems and questions why it failed to reach epidemic status at the sampling site in the Bandon estuary.

Sequencing analysis confirmed the presence of *Parvilucifera* in the North Channel, although light microscopy failed to identify it within its dinoflagellate host. Species of this parasitic genus are thought to act as generalists among dinoflagellate populations, failing to show signs of host specificity (Noren *et al.*, 2000). Yet previous studies have also suggested its main target to be *Alexandrium* (Delgado 1999; Noren *et al.*, 1999). With no direct link established between *A. minutum* and *Parvilucifera* we cannot presume it played a role in the collapse of the bloom. The only indication we have is an increased concentration of *Parvilucifera* during peak bloom periods as derived from PCR analysis. A decrease in *A. minutum* cell densities from 1.5 million cells L$^{-1}$ to 10,000 cells L$^{-1}$ within seven days in 2011 would suggest an external factor influenced the sudden bloom collapse. Encystment has been suggested as the dominant contributing factor in bloom decline (Probert 1995), and although maximum cyst densities were collected in sediment traps one week after the bloom peaks in both 2011 and 2012, they only represented ~2.5% of the vegetative population (Cosgrove *et al.*, 2014). The introduction of *P. infectans* to the water column has been proposed as a successful biological control for HABs (Noren *et al.*, 1999) based on the efficiency of its lethal infection and the fact it can be cultured in high density. But Figueroa *et al.* (2010) suggests this idea is approached with caution, as only highly susceptible strains will completely succumb to infection. They found that if the population has a parasite resistant genotype, planozygote
division was chosen over encystment allowing the more rapid production of recombinant offspring. In a separate study, *Alexandrium ostenfeldii* was found to increase its formation of the more resistant temporary cyst in an attempt to escape infection by *P. infectans* (Toth et al., 2004).

A level of host specificity was observed in the *Amoebophrya* sp. infecting the dinoflagellate populations in the North Channel. *Amoebophrya* infections were simultaneously identified within three host species in the natural samples, yet all cross infection experimentation with host cultures failed. It was thought the laboratory conditions under which the experiments were carried out may have influenced the success of the cross reactivity, yet a similar situation has been reported in other studies: Mazzillo et al. (2011) found two dinoflagellate hosts to be simultaneously infected by *Amoebophrya* in Monterey Bay, yet experimentation suggested the infecting parasite to be host specific. Isolated *Amoebophrya ceratii* dinospores infecting *A. sanguinea* cells in Chesapeake Bay also failed to infect the potential host genera *Scrippsiella*, *Ceratium* and *Gyrodinium* (Coats et al., 1996). The results presented here support the idea of *Amoebophrya ceratii* as a species complex, and suggest host specific taxa of *A. ceratii* are prevailing in the North Channel, exerting a specific level of control over each host population. A record of *A. minutum* bloom intensity in the North Channel dates back to 1996. With an average bloom density of ~40,000 cells L\(^{-1}\), *A. minutum* is often the most abundant dinoflagellate species in the water column around midsummer. Either the *A. minutum* population in the North Channel is resistant to the observed species of *Amoebophrya* similar to the case of *A. catenella* in the Thau Lagoon, France (Chambouvet et al., 2011), or it may be more susceptible to an undetected parasite. Intense blooms of *A. minutum* (>100,000 cells L\(^{-1}\)) have been found to occur every 7-8 years in the North Channel; a frequency most likely restrained by environmental conditions (Ni Rathaille et al., 2008; Cosgrove et al., 2014) and not parasitic influence.

Considering both *Amoebophrya* sp. and *P. infectans* have been detected over two summer periods in the North Channel, it leads to the question how do they survive overwinter. Cyst production as a result of sexual reproduction has not been documented in
syndiniales or Perkinsida, eliminating dormancy in the form of resting cysts as a potential survival strategy. However, an intricate relationship between *Amoebophrya* and its host has been demonstrated by Chambouvet *et al.* (2011), in which the parasite and its *S. trochoidea* host simultaneously enter dormancy. This allows the success of the parasite upon germination. We isolated 96 cysts of both *S. trochoidea* and *A. minutum* to check for signs of a parasitic infection. No unusual behavior was observed in the *A. minutum* cysts, or fluorescing green pigments in the *Scrippsiella* cysts and division followed most successful germinations. After several rounds of division, temporary cyst formation was noted in four wells originally containing *S. trochoidea* cysts. They appeared to express a green fluorescing hue which faded upon the death of the cysts. Although *Amoebophrya* doesn’t necessarily induce sexual incompetency, it does result in the host becoming reproductively incompetent (Elbrachter 1973; Nishitani *et al.*, 1985). It is therefore unlikely our observations were a parasitic infection as cysts were singly placed in each well at the beginning of the experiment. The possibility of *Amoebophrya* entering dormancy overwinter through the exploitation of its host would ensure the annual persistence of the parasite and serve as an advantageous feature when considering its introduction as a biological top-down control.

The level of parasitic control exerted on dinoflagellate bloom dynamics in an area may be partly determined by its hydrodynamics. Turbulence for example has been found to reduce the success of *Parvilucifera* infecting cells of *A. minutum* and *S. tochoidea* (Llaveria *et al.*, 2011) as it possibly lowers the encounter time between the host and parasite. As the North Channel is a sheltered and shallow system, the impact of physical factors potentially reducing the efficiency of parasitic activity are most likely lower than in an open coastal system. Studies carried out in the Baltic Sea (Gisselson *et al.*, 2002; Salomon *et al.*, 2003) and a coastal region in the Southern Atlantic (Salomon *et al.*, 2009) found *Amoebophrya* prevalence to be relatively lower than the prevalence recorded in more enclosed systems such as the Penzé Estuary (Chambouvet *et al.*, 2008) and a shallow sub-estuary of Chesapeake Bay (Coats *et al.*, 1996). This suggests tidal movements play an important role in host-parasite dynamics and in the propagation of parasitic infections within the algal community. Even in a highly retentive environment
such as the North Channel, the harmful algal species of *A. minutum* still manages to reach exceptionally high cell densities in the presence of parasitic species. The effectiveness of *Amoebophrya* in regulating its host population also has to be questioned as a prevalence rate of 77% did not manage to eliminate the *H. triquetra* population. Although it did impact upon cell densities, it appeared to inflict only short term cell reductions, as the *H. triquetra* population peaked within seven days after a 55% prevalence rate infection. If introduced, the dinospore: host ratio would need to be sufficient to ensure the maximum rate of infection. Parasitic infection has been deemed to be more effective on HAB regulation than other biological controls such as microplankton grazing (Montagnes et al., 2008). In conclusion, due to the complexity of host specificity in *Amoebophrya* spp. and the varying levels of impact both *Amoebophrya* and *Parvilucifera* spp. have upon the life-history stages of their hosts, extensive investigation exploring these topics is required before the introduction of a marine parasite as a potential top-down control is considered further.

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Chapter IV

The influence of bloom intensity on the encystment rate and persistence of *Alexandrium minutum* in Cork Harbour, Ireland

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ABSTRACT

Toxic *Alexandrium minutum* blooms recur annually in Cork Harbour, Ireland where they initiate in an inlet known as the North Channel. The dynamics of these blooms have been studied since 2003, and a high degree of inter-annual variability in the cell densities has been observed. Two intense blooms, with maximum cell densities >500,000 cells L$^{-1}$, were observed in the summers of 2004 and 2011. Annual cyst surveys during winter found that cyst densities decreased after the 2004 bloom, and by 2010 an average of ca. 40 cysts·g dry wt sediment$^{-1}$ was recorded. The intensity of blooms was found to be independent of the cyst density measured the previous winter. The cyst input to the sediment during both intense and low density blooms was measured directly through the deployment of sediment traps in the North Channel. The data allowed an estimate of the proportion of the *A. minutum* vegetative cells that underwent successful encystment, which averaged at 2.5% across a range of cell densities spanning three orders of magnitude. Maturation times of fresh cysts were determined at 5, 10 and 15 °C. The maturation time at 15 °C was found to be approximately 5 months, a value which increased by two months for a 5 degree decrease in temperature. A cyst dynamics model was constructed based on the field data to simulate the temporal variation of *A. minutum* cysts in the oxic layer of sediment. It revealed that a degree of resuspension is required to prevent cyst stocks from becoming exhausted in the thin oxic layer at the surface of the sediment. The model also demonstrated that the cysts supplied by periodic intense blooms, which occur with a frequency of every 7-8 years, are not in themselves enough to allow the population to persist over long time scales (decades). The cyst input from interim blooms of lower density is however enough to ensure the annual inoculation of the water column with *A. minutum* cells.

**Keywords:** *Alexandrium*; Ireland; bloom dynamics; cyst stocks; encystment rate
INTRODUCTION

Paralytic Shellfish Poisoning (PSP) is a potentially lethal syndrome in humans caused by the ingestion of potent neurotoxins collectively known as saxitoxins which are vectored through shellfish. These toxins concentrate in shellfish as a consequence of filter-feeding on toxin-producing microorganisms, and have had a substantial impact on cultured and wild shellfish production, often leading to harvest closures. The toxins are produced in the marine dinoflagellates *Pyrodinium bahamense*, *Gymnodinium catenatum* and species from the genus *Alexandrium*, notably the *A. tamarense* species complex (Cembella 1998) comprising *A. tamarense*, *A. fundyense* and *A. catenella* (Balech 1985). In Europe, however, *A. minutum* has a history of causing toxic blooms along the Atlantic coasts of France (Chambouvet et al., 2008), Spain (Franco et al., 1994), UK (Blanco et al., 2009) and Ireland (Touzet et al., 2008) as well as along the Mediterranean coast (Vila et al., 2005).

A characteristic of *Alexandrium* blooms is that they often recur annually in the same location (e.g. Garcés et al., 2004, Giacobbe et al., 2007). This is due to a sessile resting cyst stage, or hypnozygote, in its life cycle, which can remain dormant in marine sediments for several years, even in anoxic conditions (Anderson et al., 2005, Lewis 1988). A seasonal germination pattern has been observed in *Alexandrium* cysts with maximum excystment rates occurring in spring (Anderson, 1998; Matrai et al., 2005; Ni Rathaille and Raine, 2011; Angles et al., 2012). This germination initiates a bloom the following late spring/summer (Anderson 1997), enhancing sexual recombination and promoting the persistence of an *Alexandrium* population (Bravo et al., 2006). It is the retentive nature of a sheltered channel, bay or estuary that fosters chronic infestation with this toxic organism by creating a habitat suitable for seed stocks to be replenished, which then provide the inoculum for blooms annually. In contrast to open coastal waters, the benthic and pelagic life stages of *Alexandrium* spp. become closely linked within shallow retentive regions (Anderson et al., 2012).

A variability in the between year bloom intensity of *Alexandrium* has been recorded in several locations. For example, Anderson et al. (1994) note that in some years blooms and toxic events in the Gulf of Maine are more intense than others. Given that the year to year survival strategy of a population revolves around the replenishment
and persistence of the dormant cyst stage, cyst beds must play a central role in maintaining stocks through periods when cyst recruitment is low, or even non-existent. Wyatt and Jenkinson (1997) review the survival strategies of *Alexandrium* and argue that since gamete formation apparently occurs at relatively low cell densities \((10^2 - 10^3 \text{ L}^{-1})\); specialized traits such as motility or the use of allelopathic signaling must have evolved in order for gametes to find each other. They argue that otherwise the characteristic time-scale for the fusion of gametes would be of the order of months, even with cell densities of \(10^5 - 10^6 \text{ L}^{-1}\).

Once fusion of the gametes and formation of the hypnozygote has occurred, it is now recognized that a maturation period is required before the cysts become fully mature and ready for germination. Maturation times of the order of months have been reported for *A. minutum* and *A. tamarense* cysts (Ní Rathaille and Raine 2011), and generally increase with decreased temperatures (Anderson, 1980, B and-Schmidt et al., 2003, Ni Rathaille and Raine 2011). Cysts of *A. tamarense* from the St. Lawrence Estuary in Canada have been reported to take a year to mature at a temperature of 4° C (Castell Perez et al., 1998). In two separate studies, a substantial difference was found between the maturation times of *A. tamarense* from Cork Harbour and Perch Pond, Cape Cod (Ní Rathaille and Raine 2011, Anderson 1980). Such variability in the maturation of cyst species is potentially due to different geographic isolates having different dormancy requirements based on their ecological needs (Hallegraeff et al., 1998). Nevertheless, the excystment, growth, gametogenesis, encystment and maturation cycle is highly successful as a survival strategy in dinoflagellates such as *Alexandrium*.

Here we present nearly a decade of sampling data from Cork Harbour, Ireland. The data set illustrates the variability in the magnitude of summer blooms of *Alexandrium minutum*. The role of intense blooms in maintaining the population over time scales of years was investigated, and the relationship between the maturation time of freshly formed *A. minutum* cysts and water temperature was also determined.
METHODS

Study area

Water and sediment sampling was carried out in Cork Harbour, Ireland (Figure 1A) between the years 2003-2012. The prominent bathymetric feature in Cork Harbour is the hydrodynamically scoured channel which gouges through an otherwise relatively shallow harbour, passing Cobh and continuing up to Cork city. A shallower (5-10 m deep) channel traverses the area to the east, flows past East Ferry, and continues into the North Channel, where it then turns to the west and depths shallow from 10 m to less than 4 m at the western end of the channel. The North Channel (Figure 1B) is approximately 9 km long (east-west) and between 0.5 and 1 km wide. To either side of the channel, water depths vary up to approximately 3 m at mid-tide, and an extensive area of mudflats becomes exposed at low tide. The mudflats constitute a significant (44%) proportion of the total area. The mean tidal range of the North Channel measured at Cobh is 3.7 m (spring tides) and 2.0 m (neap tides), with a tidal excursion of 6-8 km. As a consequence, the water in the western end of the North Channel is retentive and it is here that *Alexandrium* blooms initiate and the highest cyst densities of *A. minutum* are found (Ni Rathaille and Raine 2011). There is little or no mixing between water in the North Channel and Lough Mahon areas. These waters meet at a pronounced salinity front at 8 18.2’W near Belvelly Bridge at high tide, and this region dries out at low tide (Ni Rathaille 2007). Particular attention was made to sampling the North Channel area.
Environmental data collection

Water temperature was recorded hourly with a Ti-dbiT data logger (Onset, Maine) attached to a mooring deployed in the middle of the North Channel between stations O and N-O (Fig 1B). The data set is continuous throughout the entire period except for seven months between April and December 2009 when the record was lost due to battery failure, after which the sensor was upgraded from v1 to v2. These measurements were supplemented with data for temperature and salinity made with a WTW iT90 temperature salinity probe. Incident photosynthetically active radiation was measured with a Licor LI190 quantum sensor, mounted 3 m above ground level in a shade free environment on the Northern shore of the North Channel. Data were logged hourly with a LI1000 data logger. This data record was not however continuous throughout the year, and was supplemented with global solar radiation measured using a pyranometer at the weather station at Valentia (51 54’N; 10 21’W) operated by Met Eireann.

Alexandrium cell identification and enumeration

Water sampling was routinely carried out during the months of May to September, with a frequency of at least once per week. The samples were taken along a transect of five stations in the North Channel (Figure 1B) at intervals of ca. 0.5 km, and occasionally at other locations in the estuary. At each station, surface (bucket) and sub-surface (Niskin water bottle) samples were collected. Sub-samples for the enumeration of Alexandrium were stored in 50 mL tissue culture bottles and preserved with either 0.4mL of Lugol’s iodine or 1mL of 20% buffered formaldehyde solution (final concentration 0.8%). Samples were kept at room temperature in the dark until enumeration was carried out using inverted microscopy (McDermott and Raine 2010). From 2006 onwards additional samples were taken for enumeration by fluorescent in situ hybridisation (FISH). These were collected by pre-filtering a 2000 mL water sample through a 150 µm mesh sieve and collecting on a 47mm diameter 5 µm mesh filter. All material collected on the filter was backwashed into 50mL polypropylene centrifuge tubes using 0.22 µm filtered seawater (FSW). Samples were fixed with formalin (1% final concentration v/v) and kept in the dark for up to 6 hours. The preserved samples were then centrifuged to a pellet (4000g, 10 min) and re-suspended in FSW, a process repeated three times to remove any residual formalin.
The pellet was finally resuspended in 14 mL ice-cold methanol and stored at -20 °C until analysis.

Prior to 2006, *Alexandrium* cells were identified to species level using Calcofluor white (Fritz & Triemer 1985). Phytoplankton samples were concentrated through settlement in an Utermöhl settling chamber (HydroBios, Kiel) and calcoflour white (0.05 mL of 3% w/v solution) was added to the 3 mL remaining in the base chamber. *Alexandrium* species were then examined at a magnification of x200/x400 using an inverted microscope (Olympus CKX-41) fitted with a 100W epi-fluorescence lamp using a UV filter set. Cells were identified as either *A. tamarense* or *A. minutum* based on the shape of the posterior sulcal plate. An acupuncture needle was used to rotate the cells to observe thecal plate patterns where necessary. The FISH method, based on that described by Miller and Scholin (1998), was used from 2006 to differentiate between *A. minutum* and *A. tamarense* using the probes MinA and TamB (Touzet et al., 2008). Both probes were hybridised to an aliquot (0.5-1 mL) of methanol suspension, one with the fluorohrome CY-3 (*A. minutum*) and the other with FIT-C (*A. tamarense*), filtered onto a polycarbonate filter, washed and then 5 µL of Calcofluor (100 µg mL⁻¹) was added to the filter. This allowed the analysis of armoured dinoflagellates and discrimination between the *Alexandrium* species under the same field of view, using a three position slider filter system for Calcofluor (UV) and the two fluorochromes on the inverted microscope. All details of this method, including optical filter characteristics can be found in Touzet and Raine (2007).

**Sediment sampling and cyst enumeration**

Sediment samples were collected during 1-2 surveys carried out each winter between October and February 2003-2012, with the exception of 2009. Sub-tidal sediment samples were obtained using a manually operated Ekman Birge bottom sampler (HydroBios, Kiel). Triplicate sub-samples of surface sediment were taken from each sample using 50 mL syringes which had the end sawn off. These were filled manually to the depth of the sediment in the sampler by simultaneously pushing the corer down into the sediment but holding the plunger static. This ensured no air entered the sample. The syringe was then withdrawn and the end sealed with Parafilm. Intertidal samples were collected from the shoreline in triplicate by
sampling directly into the sediment. All sediment cores obtained were kept in the dark at 4° C until analysis.

In order to quantify the number of cysts, the top (0-1) cm of each sediment core was removed, mixed to ensure homogeneity, and a weighed volume (usually 0.4 mL) was transferred into a 100 mL glass Schott bottle containing 40 mL of FSW and then ultrasonicated for 3 minutes at 23kHz. The sample was sieved through an 80 µm sieve and collected on a 20 µm sieve before being backwashed into a 15 mL centrifuge tube. The cysts were extracted using density gradient centrifugation with sodium polytungstate. Prior to 2007, Ludox was used for the density gradient (Ní Rathaille 2007). Sedgwick-Rafter cells were used to enumerate *A. minutum* cysts at x100 magnification using an Olympus CK-41 microscope. The water content of the sediment was recorded by weighing an aliquot of sediment before and after drying to constant weight at 80 °C, allowing presentation of cyst densities as cysts mL⁻¹ and (g dry wt.)⁻¹. Vertical profiles of *Alexandrium* cysts in the top 5 cm of sediment were obtained on occasion by counting the cyst densities in 1 cm sections along a core.

**Encystment and maturation rates**

Sediment traps were deployed at two locations in the western half of the North Channel at stations NO and O (Figure 1B) during the summer periods of 2004, 2011 and 2012 and one location in 2006. The traps consisted of cylindrical tubes 0.6 m long, 10.5 cm ID and were fitted with baffles near the top to reduce turbulence over the surface. Each trap was emptied on a weekly basis. Once the particulate material settled (ca. 1.5 hrs), the supernatant was removed and the volume of collected sediment was noted. The samples were then stored at 4° C in the dark until analysis. Cysts were extracted from a sub-sample of measured sediment and enumerated using the methods as described in 2.4 above. This allowed an estimate of the total cyst input to the sediment over the seven day collection period.

The encystment rate, or cyst yield, was estimated by dividing twice the numbers of cysts caught in the traps following the period of peak vegetative cell intensity by estimated numbers of cells above the trap during the bloom peak. As the formation of a cyst requires the fusion of two gamete cells, the number of cysts collected in the traps was divided by two. Thus if the peak bloom intensity was N cells L⁻¹ on July 1st,
the numbers of cysts collected after this date (over the following 14 days) was \(C\) cm\(^{-2}\),
and the water height above the sediment traps was \(h\) meters, then the encystment rate
\((\text{En})\) would be \((2 \times C)/(N \times h)\):

\[
\text{En} = \frac{(2 \times C) \times 10000}{(N \times h) \times 1000} \times 100
\]

We chose to use the cyst densities collected in the two week period after the bloom
peak as the length of time required for a vegetative cell to form a hypnozygote cell is
thought to be 7-14 days. Using the vegetative cell density during the bloom peak to
calculate the encystment rate also increases the accuracy, due to a higher magnitude
of cells in the water column. This calculation assumes all cysts collected in the
sediment traps are freshly formed cysts in the water column. There are several
uncertainties and potential errors associated with this method of calculation, as with
other ways of estimating encystment that have been used. These are discussed later.

Three stocks of sediment collected from the sediment traps one week after peak cell
intensities were observed in 2011 were sub-sampled into airtight containers and stored
in the dark at 5 °C, 10 °C and 15 °C. Every 30 days, 30 cysts were isolated from
each of these and placed into individual wells of a 96 well plate with 250 µl of FSW.
Each plate was then stored at the temperature corresponding to that of the cyst storage
under an irradiance of 100 µE.m\(^{-2}\).sec\(^{-1}\) and a 14:10 light: dark cycle. The cysts were
examined for signs of germination every 15 days. Maturation times were determined
from the time required for 50% of the cysts to germinate (Binder and Anderson,
1987).

**Excystment rate**

Excystment rates were calculated from the seasonal excystment pattern of \(A.\ minutum\)
cysts presented in Ní Rathaille and Raine (2011). As only the top 2 mm of sediment
was oxic, an environmental condition required for germination (Anderson \textit{et al.},
1987), only 20% of the \(Alexandrium\) cyst density in the top 1 cm of sediment was
used). The number of cysts \((N_t)\) remaining after a timestep \(dt\) was calculated using
Equation 1 (Anderson \textit{et al.}, 1987) where \(E_x\) is the seasonal excystment rate

\[
N_t = N_{t-1} \times e^{(-E_x dt)}
\]
An *Alexandrium* cyst dynamics model was constructed to simulate the temporal variation of *A. minutum* cysts in the sediment surface. Cyst input was estimated from encystment rates as calculated above. Excystment from the oxic layer of sediment in the North Channel was estimated using the initial cyst density on day 1 (January 1st) from 20% of the top 1 cm observed during the winter survey of 2003/2004 applying an average over 13 stations. It was quickly apparent that cysts in the oxic layer were exhausted rapidly and therefore a monthly resuspension event was incorporated into the model to represent the natural replenishment of cysts from the 8 mm anoxic layer below.
RESULTS

Alexandrium minutum bloom dynamics

Figure 2A presents a typical temperature-salinity diagram for water in Cork Harbour. The data clearly show the lack of mixing between waters in the eastern and western sections of the estuary, and the absence of mixing between water at the western end of the North Channel and that of the Lough Mahon area of the main channel leading to Cork City. Figure 2B illustrates the daily mean water temperature in the North Channel from January to December between the years 2003 - 2012, ranging from 6.8 °C in January to 18.1 °C in August, and Figure 2C the mean incident global irradiance, measured at the Valentia Observatory in 2004. The irradiance data have been converted to average hourly water column photosynthetically active radiance (PAR), based on a 14 hour day and using a mean water column attenuation on the secondary axis (see Ní Rathaille et al., 2009 for details). Figure 2D shows the output from the PAR sensor located at the North Channel, where again the incident irradiance has been converted to mean hourly depth-averaged sub-surface irradiance.

Figure 2. Environmental parameters. A) A typical temperature and salinity plot from Cork Harbour, showing a lack of mixing between water in the North and West Channels, emphasizing the retentive nature of water in the North Channel. B) Mean daily sub-surface temperature from 2003-2012 recorded at the temperature sensor mooring (for location see Figure 1b). +/- standard deviation is also shown; C) Daily global solar radiation measured at Valentia, Co. Kerry for 2004. The primary y-axis indicates mean hourly underwater irradiance levels for a 2 metre water column calculated from the solarimeter data (see text). D) Mean hourly underwater irradiance (PAR) levels calculated from incident PAR for 2011.
Temperature and daily average water column PAR cease to have an effect on the growth rate of *A. minutum* above 15 °C and 100 μE·m⁻²·s⁻¹ respectively (Ní Rathaille et al, 2009) across the ranges experienced in Cork Harbour. The balance between tidal dilution and combined temperature and PAR controlled growth has allowed the successful prediction of *Alexandrium* bloom initiation in the North Channel (Ní Rathaille 2007), and typically limits blooms to the four month period between mid-May to mid-September.

*Alexandrium* cell densities within the North Channel between the years 2004 and 2012 are shown in Figures 3 and 4. The values shown are a weekly average across all five water stations sampled at the surface. The average water depth during sampling was ~8 m at station R and 5 m at station NO. In each year, the blooms developed after the first (large) spring tide in June, as the spring tide which follows is relatively smaller due to the summer solstice. This reflects the ability of *A. minutum* to increase when tidal dilution rates are, on balance, lower than in the spring or autumn. The *Alexandrium* cell densities increased during June and the blooms lasted for approximately two weeks. Figure 3 illustrates two intense blooms, defined here as cell densities in excess of 100,000 cells L⁻¹, which occurred in June 2004 when cell densities reached a maximum of $1.7 \times 10^6$ cells L⁻¹ and June 2011 when cell densities reached $1.5 \times 10^6$ cells L⁻¹. In other summers, the maximum recorded *Alexandrium* levels were 10,000 - 80,000 cells L⁻¹ (Figure 4A-F). In 2012, two successive relatively small blooms occurred. This sequence was most likely also controlled by tidal dilution, as both blooms followed a small spring tide on either side of a much larger one (Figure 4F). No water samples were collected during the summer of 2009.

Figure 3. *Alexandrium* vegetative cell densities averaged over five water sampling stations during the summers of 2004 and 2011 plotted with tidal dilution. The numbers of *A. minutum* cysts caught in sediment traps each week through the blooms are also shown as the total cyst input cm⁻² for the duration of the trap deployment (one week). Vertical downward arrows indicate the first spring tide in June.
Chapter IV

Figure 4. *Alexandrium* vegetative cell densities averaged over five water sampling stations during the summers of 2005-2012 and plotted with tidal dilution are shown in figures 4A-F. Vertical downward arrows indicate the first spring tide in June, and it can be seen that all blooms, with the exception of that in 2012, developed after this event.

*Alexandrium minutum* cyst dynamics

Table 1 gives the *A. minutum* cyst densities in Cork Harbour measured in the top 1 cm of sediment core samples taken during three cyst surveys carried out in the winter of 2003-2004. Cyst densities were significantly different (p<0.01) in each of the three regions, delineated geographically in Figure 1A, with highest levels in the North Channel. Cyst densities were approximately one third of these densities in the area to the south of the North Channel, but were over an order of magnitude lower in the main western region of Cork Harbour.

Table 1. *A. minutum* cyst densities, Cork Harbour, Oct-03 to Mar-04. Densities are expressed in both volume and dry weight basis for the top 1 cm layer of sediment. The areas outside the North Channel are indicated in Figure 1A.

<table>
<thead>
<tr>
<th>Area</th>
<th>Range  (cysts·ml⁻¹)</th>
<th>Mean   (cysts·ml⁻¹)</th>
<th>sd (n)</th>
<th>Range  (cysts·g dwt⁻¹)</th>
<th>sd (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Channel</td>
<td>75-1370</td>
<td>270</td>
<td>190 (25)</td>
<td>130-1680</td>
<td>245 (25)</td>
</tr>
<tr>
<td>South of East Ferry</td>
<td>17-230</td>
<td>115</td>
<td>60 (9)</td>
<td>19-240</td>
<td>61 (9)</td>
</tr>
<tr>
<td>Western Section</td>
<td>4-25</td>
<td>24</td>
<td>14 (9)</td>
<td>10-95</td>
<td>29 (6)</td>
</tr>
</tbody>
</table>
Table 2. Annual observed *A. minutum* cysts· g dry wt sediment\(^{-1}\) and in cysts mL\(^{-1}\) (SD values in parentheses) alongside the bloom intensity of *A. minutum* vegetative cells L\(^{-1}\). The modeled cyst density (mL\(^{-1}\)) at the start of each year is shown for three scenarios: using a simple 2% encystment rate from the observed bloom, with no intense blooms in 2004 and 2011 where the bloom intensity has been artificially suppressed at 20,000 cells L\(^{-1}\), and with cyst inputs from intense blooms alone.

<table>
<thead>
<tr>
<th>Year</th>
<th>Observed Cyst density (g dwt(^{-1}))</th>
<th>Observed Cyst density (mL(^{-1}))</th>
<th>Observed bloom intensity (Cells L(^{-1}))</th>
<th>Observed bloom intensity (Cysts mL(^{-1}))</th>
<th>No intense bloom yrs (Cysts mL(^{-1}))</th>
<th>Intense bloom yrs only (Cysts mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>345 (245)</td>
<td>270 (190)</td>
<td>470000</td>
<td>270</td>
<td>270</td>
<td>270</td>
</tr>
<tr>
<td>2005</td>
<td>130 (95)</td>
<td>80 (60)</td>
<td>30000</td>
<td>1269</td>
<td>69</td>
<td>1269</td>
</tr>
<tr>
<td>2006</td>
<td>115 (60)</td>
<td>56 (35)</td>
<td>80000</td>
<td>194</td>
<td>67</td>
<td>134</td>
</tr>
<tr>
<td>2007</td>
<td>-</td>
<td>-</td>
<td>20000</td>
<td>181</td>
<td>167</td>
<td>14</td>
</tr>
<tr>
<td>2008</td>
<td>-</td>
<td>-</td>
<td>30000</td>
<td>59</td>
<td>58</td>
<td>2</td>
</tr>
<tr>
<td>2009</td>
<td>45 (35)</td>
<td>35 (25)</td>
<td>30000</td>
<td>66</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td>2010</td>
<td>80 (40)</td>
<td>35 (18)</td>
<td>3000</td>
<td>67</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td>2011</td>
<td>45 (40)</td>
<td>11 (7)</td>
<td>800000</td>
<td>13</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>2012</td>
<td>1070 (760)</td>
<td>760 (450)</td>
<td>30000</td>
<td>1661</td>
<td>41</td>
<td>1660</td>
</tr>
<tr>
<td>2013</td>
<td>150 (120)</td>
<td>145 (120)</td>
<td>-</td>
<td>236</td>
<td>64</td>
<td>176</td>
</tr>
</tbody>
</table>

*A. minutum* cyst densities in the North Channel in this and subsequent winters up to that of 2012/2013 are shown in Table 2. It should be noted that no *A. minutum* cyst data is available for the winter periods of 2006/07 and 2007/08. It was quite evident that densities decreased after 2004, and by the winter of 2010 cyst densities were averaging ~45 cysts· g dry wt sediment\(^{-1}\). In December of 2011, following the intense summer bloom.
of 2011, cyst densities had increased by 1000 cysts·g dry wt sediment\(^{-1}\). The vertical distribution of \textit{A. minutum} cysts within the cores was analysed in order to check that the variation in the cyst data was not due to vertical heterogeneity within the sediment. Profiles are shown in Figure 5 where it can be seen that in the winters following intense blooms (2004, 2011) most of the cysts are in the top 1-2 cm. Cysts appear more homogeneously distributed otherwise (e.g. 2003, 2006; Figure 5). At a depth of 4-5 cm the cyst density was 5-30 cysts·g dry wt sediment\(^{-1}\). Cyst densities at each depth interval are represented as a percentage of the total number of \textit{A. minutum} cysts in each core in order to portray on a relative basis the inter-annual variance from 0-5 cm.

The modeled cyst density (mL\(^{-1}\)) at the start of each year is shown for three scenarios: using a simple 2\% encystment rate from the observed bloom, with no intense blooms in 2004 and 2011 and with cyst inputs from intense blooms alone.

The number of \textit{A. minutum} cysts caught in sediment traps deployed in 2004, 2006, 2011 and 2012 are shown in Table 3, along with the observed levels of vegetative \textit{Alexandrium} cells. The cysts were very similar to the mature diploid hypnozygotes observed in sediments of the North Channel taken in winter. They typically presented a spherical shape, but slightly ellipsoidal in lateral view, and ranged in size from 15-30 µm. The contents were characteristically comprised of numerous pale green lipid globules and a single orange accumulation body (Figure 6). Encystment rates were derived from the cyst numbers caught in the sediment traps and the vegetative cell density (Table 3). They ranged in values from 0.7 to 5.1\% of the vegetative cell densities observed during the summers when the traps were deployed in the western half of the North Channel (stations O, NO) 2004, 2006, 2011 and 2012. The mean encystment rate was 2.5\%.
Table 3. *A. minutum* bloom intensity (cells L\(^{-1}\)) and total cysts cm\(^{-2}\) collected by the sediment traps following the bloom peak during the summers of 2004, 2006, 2011 and 2012. Two bloom peaks occurred in 2012. Locations of the traps were in the proximity of sampling stations as indicated in figure 1B.

<table>
<thead>
<tr>
<th>Year</th>
<th>Trap collection period</th>
<th>Location</th>
<th>Mean water height above trap (m)</th>
<th>Total cyst input (cm(^{-2}))</th>
<th>Bloom intensity (cells L(^{-1}))</th>
<th>Encystment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2004</td>
<td>Trap 1 16 June-30 June</td>
<td>R</td>
<td>7</td>
<td>2490</td>
<td>620000</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Trap 2 16 June-30 June</td>
<td>NO</td>
<td>4</td>
<td>6350</td>
<td>620000</td>
<td>5.1</td>
</tr>
<tr>
<td>2006</td>
<td>Trap 1 22 June-29 June</td>
<td>O</td>
<td>2.5</td>
<td>70</td>
<td>80000</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Trap 2 29 June-13 July</td>
<td>NO</td>
<td>4</td>
<td>5200</td>
<td>830000</td>
<td>3.1</td>
</tr>
<tr>
<td>2011</td>
<td>Trap 1 29 June-13 July</td>
<td>NO</td>
<td>2.5</td>
<td>1800</td>
<td>830000</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Trap 2 29 June-13 July</td>
<td>O</td>
<td>4</td>
<td>125</td>
<td>30000</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Trap 1 30 May-20 June</td>
<td>NO</td>
<td>2.5</td>
<td>110</td>
<td>30000</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Trap 2 30 May-20 June</td>
<td>O</td>
<td>2.5</td>
<td>255</td>
<td>40000</td>
<td>3.2</td>
</tr>
<tr>
<td>2012</td>
<td>Trap 1 20 June-11 July</td>
<td>NO</td>
<td>4</td>
<td>80</td>
<td>40000</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Maximum numbers of cysts in the traps were recorded spanning the week after the peak vegetative cell density was observed (Figure 3A, B). An additional deployment in 2004 at the eastern end of the channel, north of station R (Figure 1), recorded a lower percentage of cysts (Table 2). The maximum observed cyst input rate to the sediment was 5200 cysts cm\(^{-2}\) in 2011, over an order of magnitude higher than the maximum recorded in 2012 (255 cysts cm\(^{-2}\)). The increase in cyst densities observed in the winter of 2011 was approximately 1500 cysts g dry wt sediment\(^{-1}\), or 760 mL\(^{-1}\). This value compares quite favorably with estimates of cyst input made directly from the two sediment traps, which collected between 1800 and 5000 cysts cm\(^{-2}\) over the entire bloom, and installs a degree of confidence in both sets of field measurements.

Figure 7 shows the effect of temperature on the maturation of fresh cysts caught in the sediment traps. Maturation times, defined as the time taken for 50% of cysts to excyst, were 160 days at 15 °C, 225 days at 10 °C and ~280 days at 5 °C. The results
indicated that an increase in 5 °C decreases maturation times by approximately two months.

Figure 7. Results of a 10 month maturation experiment carried out on freshly formed *A. minutum* cysts collected in a sediment trap deployed in the North Channel of Cork Harbour. The cysts were stored at 5 °C, 10 °C and 15 °C. The date of cyst collection is indicated on the figure.

*Alexandrium minutum* cyst dynamics model

A cyst dynamics model was run using the data and parameters deduced from the field study. Given the long maturation times of *A. minutum* cysts (Figure 7), coupled with observed low levels of germination in the months of September-January (Ni Rathaille and Raine, 2011) fresh cysts were deemed not to germinate in the year of their formation. Germination rates were derived from the seasonality observed by Ni Rathaille and Raine (2011) where the numeric values portrayed in Figure 8A were used. An example output of cyst densities in the sediment through a year is shown in Figure 8B. The model output is shown in Table 2, and shows a good correspondence between observed and modeled cell densities when an encystment rate, more accurately defined as the rate of cyst input to the sediment of the North Channel, of 2% of the vegetative population is applied. Statistical treatments of the model output indicate the lowest variance when an encystment of 1-2% was applied (Figure 8C). The model output showed that cyst densities in the sediments became unrealistically high when applying rates of 10% or more. The model was tested for sensitivity against the depth of the oxic layer of the sediment but there was no significant difference in the relationship between observed and modeled winter cyst densities when applying a value of either 2 or 3 mm.
The model was also run to test the hypothesis that periodic intense blooms are essential to maintain a population of *A. minutum* in the North Channel over decadal scales. The model was first run with observed bloom intensities from 2004-2012 using an encystment rate of 2%. The model was then run with smaller blooms (20,000 cells L$^{-1}$) substituted for the intense blooms of 2004 and 2011. In both of these runs, cyst stocks were maintained over the 9 year period (Table 2). Finally, it was used to test the hypothesis that populations can persist with cyst input deriving from intense blooms alone. The model output clearly showed that over the time scale of the frequency of these blooms, every 7 years, stocks become exhausted (Table 2). The effect of variation in smaller bloom intensity was investigated in tandem with the encystment rates 1, 2 and 3%.

Figure 8. Excystment dynamics of *A. minutum*. A) The seasonal variation in the excystment rate in cysts of *A. minutum* (adapted from data presented in Ní Rathaille and Raine 2011), showing the interpolated values used in the cyst dynamics model (see text). B) A model simulation of the seasonal excystment of *A. minutum* over 12 months in the surface 2 mm thick oxic layer of North Channel sediments with and without resuspension of sediment. No inputs of fresh cysts were applied. Note the rapid exhaustion of cysts if no replenishment through resuspension occurs. C) Plot of the correlation ($r^2$) between observed *A. minutum* cyst densities and those derived from the output of a cyst dynamics model when the encystment rate is changed, based on observed cell densities in the water column. D) Steady state winter sediment cyst densities (cysts mL$^{-1}$) which result from the output of the cyst dynamics model which is artificially forced with recurring annual blooms of 1000, 10,000 and 20,000 cells L$^{-1}$ intensity.
Summer blooms were held at relatively low levels (1000, 10,000 and 20,000 cells L$^{-1}$). In each case, the system ran to a steady state as regards the winter cyst density (Figure 8D). The conclusion is therefore that intense blooms are not necessary to maintain stocks of *A. minutum* in Cork Harbour, and the cyst bank will persist over the decadal time scales observed.

**DISCUSSION**

The North Channel of Cork Harbour contains a mixture of *Alexandrium* species: *A. minutum*, *A. tamarense* (Gp.III, Lilly *et al.*, 2005), and *A. ostenfeldii* (Touzet *et al.*, 2011). Toxins are produced by *A. minutum* (principally GTX2 and GTX3, Touzet *et al.*, 2008) and *A. ostenfeldii* (spirorides, Touzet *et al.*, 2008). Planktonic cells of *A. minutum* can be found each summer in water samples taken from the North Channel, while a toxic bloom of this species inhibits the harvesting of shellfish produced locally. It is therefore appropriate to define the term bloom with regard to this species. We use the terms ‘bloom’ to signify the presence of cells in the water, ‘toxic bloom’ where the cell density is high enough to contaminate shellfish with toxins above the maximum permissible level (MPL: 80µg 100g flesh$^{-1}$; EC regulation 853/2004), and ‘intense bloom’ when densities of over 100 *10$^3$* cells L$^{-1}$ occur. Misinterpretation of the term ‘bloom’ (see e.g. Smayda, 1997) is avoided by defining *Alexandrium* blooms in the North Channel this way. *A. minutum* blooms are also known to recur annually around June in the Penzé Estuary in Brittany, France (Andrieux–Loyer *et al.*, 2008), yet in contrast to the North Channel, cell densities of up to 1 x 10$^6$ cells L$^{-1}$ are required for a toxic event (Chapelle *et al.*, 2010). This is due to the large tidal reach in the Penzé Estuary, which is 28km in length. As a consequence, the contact time between the bloom, usually found in a restricted range of salinity, and the shellfish is reduced. A high density of *A. minutum* is thus required for a toxic event, one to two orders of magnitude higher than in the North Channel of Cork Harbour.

Records of annual monitoring in Cork Harbour dating back to the 1990’s (Silke 1998) show that intense blooms of *Alexandrium* occur with a frequency of once every 7-8 years. The precise speciation of an intense bloom in 1996 of up to 845,000 cells of *Alexandrium* spp. L$^{-1}$ is unavailable (Silke, 1998). It is reasonable to assume that *A.
minutum comprised a significant part of the Alexandrium biomass as it was associated with a PSP event where GTX2 and GTX3 were the causative toxins. These toxins are characteristic of A. minutum in Cork Harbour (Touzet et al., 2008). No records of Alexandrium cell densities above 30,000 cells L\(^{-1}\) exist until 2004, when a bloom peak of 1.7 x 10\(^6\) cells L\(^{-1}\) comprised ~80% A. minutum. In the intense bloom of 2011, FISH results confirmed >99% of the Alexandrium cell population was that of A. minutum. Clearly there is sufficient seed (cyst) stock to allow perpetuation of annual A. minutum blooms over decades.

A degree of variability was found in both the horizontal and vertical distribution of cysts within the sediment of Cork Harbour. Wyatt (2002) draws a hypothetical analogy between the seed shadow of terrestrial plants with the distribution of dinoflagellate cysts in marine sediments. Both would be influenced by the speed, direction and turbulent characteristics of the environmental medium, air in the case of trees and seawater for dinoflagellate cysts. Over the sampling period in Cork Harbour, we have observed four locations where cysts were found in significantly higher densities compared to other regions sampled on the same day (Cosgrove et al., 2010). All of these could be related to local hydrography: in a lagoon area off the north shore, at the location of gyres which are generated with flood and ebb tides to the north and south of the channel immediately west of the entrance to East Ferry, and at the western end of the North Channel. Each of these became routine sampling sites in annual cyst surveys, along with 8 other sampling stations in the North Channel, and allows a degree of confidence in the temporal data set which shows a general decrease in cyst densities between the winters of 2004/5 and 2010/11. Vertical cyst profiles taken during the winter periods of 2004 and 2011 show a large percentage of cysts residing within the top 1cm of sediment, a result which can be related to post bloom situations. Blooms in both 2003 and 2006 were quite low in intensity, and as a consequence cyst distribution profiles taken during both subsequent winter periods show a relatively homogenous pattern from 0 to 5 cm. In the vertical cyst profile of 2012, the highest percentage of cysts was found within the 1-2 cm depth range, which may reflect a degree of burial of the cyst stock created during 2011.

The low winter cyst density measured in October 2010 provides clear evidence that the intensity of spring/summer Alexandrium blooms in the North Channel is
independent of the cyst density measured the previous winter. Although the intense bloom in 2004 followed cyst densities of \( \sim 300 \) cysts g\(^{-1}\), cyst densities were only \( \sim 40 \) cysts g\(^{-1}\) in the winter prior to the intense bloom in 2011. A study by Estrada et al. (2010) showed excystment fluxes to have little impact on bloom magnitude following its inoculum, supporting the hypothesis that the magnitude is independent of cyst density. The initiation of *Alexandrium* blooms in the North Channel of Cork Harbour can be successfully modeled using the balance between tidal dilution and the in situ growth rate derived from light and/or temperature control (Ní Rathaille 2007). Temperature ceases to control growth above 15 °C whereas irradiance ceases to control growth once daily average levels of underwater irradiance exceed 100 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) (Ní Rathaille et al., 2009). The bloom magnitude appears to be influenced by the variability in sub-surface irradiance (Ní Rathaille et al., 2008). Both intense blooms of 2004 and 2011 occurred during periods of sustained high irradiance levels. This finding is in contrast to a study carried out in the Gulf of Maine in which a coupled physical/biological model found cyst abundance to be the most important factor capable of influencing the bloom intensity of local *A. fundyense* populations (He et al., 2008).

A consistent observation was that maximum cyst deposition (as measured by sediment traps in 2004, 2006, 2011 and 2012) was observed approximately one week after high cell densities were observed. This time scale, equivalent to the sediment trap collection period, is of a similar magnitude to that of the progression of planozygotes to cyst formation in *A. minutum* cells (Figueroa et al., 2007). Therefore the cell density from which the proportion of encystment calculated was that of the peak bloom intensity. There is more than one way of using data on vegetative cells and cyst production to calculate encystment rate (Lewis 2002). It would appear, however that using data for both variables collected on the same date, or even the mean cell concentration over the period prior to trap collection, can give unrealistically high encystment rates (Garcés et al., 2004; Angles et al., 2012).

Encystment rates for *A. minutum* of the order 0.7-5.1%, averaging at 2.5 %, of the vegetative cell population were measured directly through the use of sediment traps. This proportion represents that part of the population ending up as (hypnozygote) cysts in North Channel sediments. It does not reflect the rate of gametogenesis, which
we were unable to measure, and is likely to be an underestimate due to factors such as tidal flushing exporting a proportion of planozygotes and fused gamete pairs. Probert (1999) reported that planozygotes made up 16% and 47% of motile *A. minutum* cells around the time of bloom maxima in the Aber Wrac’h and Penzé estuaries respectively, and similar proportions of planozygotes have been observed in blooms of *A. tamarense* (Anderson *et al.*, 1998). Cysts were present in the sediments of Cork Harbour immediately outside the North Channel which may reflect the export of planozygotes. Nevertheless, when a sediment trap was deployed at the eastern end of the North Channel in 2004, cyst numbers caught in these were substantially reduced relative to those deployed in the western end, and one would expect this decreasing trend to continue outside the study area.

Garcés *et al.* (2004) present data on the dynamics of an *A. minutum* bloom which occurred in Arenys del Mar harbour, Spain in 2002. They show using sediment traps that ~1% of the vegetative cells ended up as cysts caught in sediment traps. The bloom intensity was 50 * 10⁶ cells L⁻¹ and lasted for approximately 3 weeks. This result is comparative with that (2.5%) derived from our observations on *A. minutum* bloom dynamics in Cork Harbour. One aspect of the use of sediment traps in shallow water columns is that they will trap resuspended material. The absence, or very low numbers, of cysts caught in the traps outside the bloom periods would suggest that cysts are not significantly resuspended into them, and do not affect the overall result. Sediment traps also tend to overestimate the vertical flux of particulate material as a consequence of turbulence across the collection opening biasing the input of particles. Nevertheless the implication is that the rate of cyst supply to the sediment is independent of vegetative cell density over a range of three orders of magnitude from 5*10⁴ to 5*10⁶ cells L⁻¹. It is concluded that blooms of intense magnitude are not an *a priori* condition for the successful collision and fusion of *A. minutum* gametes and, in turn, the encystment of planozygote cells. Models of planozygote formation resulting from the collision of gametes, based on a function of nearest neighbor distance, would imply that gamete fusion rate increases with increasing cell density (Wyatt and Jenkinson, 1997). This trend would increase independently of the success of fusion after a collision. Clearly, this is not the case for *A. minutum*. It is pertinent to note that in a similar study on *A. fundyense* in Northport Harbour (NY, USA), Angles *et al* (2012) found that cysts collected in sediment traps represented 1-
2% of the vegetative population that had bloomed to $\sim 1 \times 10^6$ cells L$^{-1}$ above the traps.

Considering >95% of the *A. minutum* population failed to encyst annually during peak bloom periods, it leads to the question; where do they go? The three most often cited causes for the termination of an *Alexandrium* bloom are export, grazing and death due to parasitic infection. Tidal dilution is known to play an influential role on the phytoplankton dynamics within the North Channel of Cork Harbour. Specific tidal flushing rates, a term which can be compared directly to specific growth rate, range from 0.15 d$^{-1}$ at neap tide to 0.6 d$^{-1}$ on spring tides (Ní Rathaille, 2007). Given that *A. minutum* blooms require ~14 days to develop, then during bloom development populations must grow, or at least maintain themselves, through a spring tide. This occurs only when spring tides are at their lowest around the summer solstice. It is likely that a proportion of the sexual phase stages (fused gametes, planozygotes) are exported during times of higher dilution rates. The sequence of gamete formation, collision, fusion, and planozygote formation must take longer than simple binary fission of vegetative cells, notwithstanding that transformation of a planozygote cell to a resting cyst is not obligate for *A. minutum* (Figueroa et al., 2007). Calbet et al. (2003) found there to be a significant relationship between microzooplankton grazers and the net growth of *A. minutum* from measurements made during the 2002 bloom in Arenys del Mar, Spain. They found that grazing by microzooplankton (0.84 d$^{-1}$) was equal to and even exceeded *A. minutum* growth rates and suggested that grazing plays an influential role in bloom termination. Large tintinnids, particularly *Favella* spp., are often associated with dinoflagellate blooms (Stoecker, 2012). They can ingest toxic dinoflagellates, including *Alexandrium*, and may help in regulating toxic blooms. *Alexandrium* bloom terminations in certain small lagoons of the Po river delta were attributed to grazing by the *Favella* sp. (Sorokin et al., 1996). Over the course of this study, abundances of tintinnids have been observed to coincide with the termination of the *A.minutum* bloom in the North Channel in 2005 (Lyons and Raine, 2006), but no data are available for the other blooms.

The effect of temperature has been the most frequently investigated environmental parameter used for experiments on dinoflagellate cyst maturation. It seems that the length of dormancy of one species varies with the geographic population under
consideration, and where temperature effects are observed, they have often been linked to the environment from where the population originated (Lewis, 2002). Thus maturation times for *A. minutum* of 28 days have been reported at 18°C using cultures (Parker and Blackburn, 2000), although with no mention of how the maturation period was defined (i.e. % germination), and so it is impossible to make any real comparisons. In an earlier study of cysts from Cork Harbour, Ni Rathaille and Raine (2011) report maturation times of 2-3 months on fresh cysts which were formed in the laboratory. Longer maturation times were found in the current study. However, the maturation time of the order of months suggests that the supply of cysts from one particular year seeds the bloom in the next, and possibly subsequent, years. This aspect was incorporated into the cyst dynamics model.

Use of the cyst dynamics model produced two main findings. The first of these was that a degree of resuspension, and hence redistribution, of cysts was required within the top one layer of sediment. Otherwise, the cyst stock in the oxic layer, a condition for excystment, would become exhausted. The model simulation illustrating the loss of *Alexandrium* cysts from the top 2-3 mm of sediment due to excystment found cyst stocks to have exhausted in the North Channel by mid-April. Resuspension is an expected condition considering the average water depth and quality of sediment in this area. The model output, with monthly resuspension, showed that although cyst loss is high during peak excystment, cysts remain present in the sediment all year round. Thus if regular resuspension is coupled with the seasonal regulation of excystment, then a continuous supply of cysts will be available to provide the inoculum of planktonic vegetative cells each spring.

Of more significance is that the cyst dynamics model could provide insight as to how the *Alexandrium* populations persist. The modes investigated were periodic cyst replenishment as a consequence of intense blooms, or else a continuous, low level supply of cysts supplemented by the occasional high input of an intense bloom. The model output indicates that a periodic large input which occurs every 7-8 years, as is the frequency of intense blooms in the North Channel of Cork Harbour, is not in itself enough to perpetuate the population, despite the fact that only a low level initial inoculum can initiate a bloom: cyst stocks are exhausted within 3-5 years using encystment rates of 2%. On the other hand, the model output showed that with the
observed variability in the annual bloom intensity, in the absence of an intense summer bloom the *A. minutum* cyst stock will be able to maintain itself with the observed variability in the levels of minor blooms. Finally, our data show that a continual low level of blooms, of the order $10^3$ to $10^4$ cells L$^{-1}$, can maintain the seed bed.

**CONCLUSION**

An important result of this study is that the intensity of blooms is independent of the cyst density measured the previous winter. We therefore conclude that periodic large pulses of encystment are not essential to ensure the annual inoculation and perpetuation of *A. minutum* cells in the water column. This outcome demonstrates the need for vigilance in spreading *A. minutum* populations. Currently, this species does not inhabit the coastal zone of North America. The likelihood of increasing the range of this organism on a global scale through anthropogenic activities such as transfers of cysts with ship’s ballast water has already been highlighted (Hallegreaff, 1998). On the other hand, its requirement for a retentive environment would reduce the number of sites where it can promulgate.

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Chapter V

Modelling the initiation and termination of *Alexandrium minutum* blooms in Cork Harbour, Ireland

Planned submission for: Journal of Marine Systems

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ABSTRACT

A greater understanding of the life-history stages of toxin producing species such as *Alexandrium minutum* increases the predictive ability of both the initiation and termination of harmful algal blooms. In addition to the influence of environmental parameters on bloom initiation, the influence of life-cycle transitions on *A. minutum* bloom dynamics are investigated in this study, focusing particularly on gametogenesis and planozygote formation. A simple ecological model based on temperature, irradiance and tidal dilution was used to simulate the initiation of the two largest blooms (>100,000 cells L⁻¹) of *A. minutum* observed in Cork Harbour since 2004. Bloom termination was then simulated using varying rates of gamete formation and gamete fusion. The accuracy of the model was tested by hindcasting the results to observed cell density data. For both intense bloom years, 2004 and 2011, a gamete formation rate of 40% was found to produce the best-fit model output. Planozygote cells were observed within natural samples in 2011 composing on average 18% of the population, while an average of only 14% were estimated to progress to encystment. A five day period between planozygote formation and encystment is suggested by the model output. This study illustrates, through the application of a simple box model, that tidal dilution can greatly influence bloom development, while sexuality has the potential to contribute substantially to bloom termination.

**Keywords:** *Alexandrium minutum*; life-history stages; bloom development; bloom termination; ecological modelling
INTRODUCTION

Species of the genus *Alexandrium* have stages in their life cycles which influence their distribution, abundance and persistence in coastal ecosystems. The formation of dormant resting cysts following sexual reproduction allows *Alexandrium* spp. to survive periods of unfavourable environmental conditions, and promotes the persistence of the population within confined water bodies over decades. In the past, *Alexandrium* life-cycle studies have focused primarily on the relationship between the vegetative population and the hypnozygote (cyst) stage (Ishikawa and Taniguchi 1996; Garcés et al., 2004; Estrada et al.; 2010, Cosgrove et al.; 2014), while less research has been directed towards the intermediate stages of sexual reproduction, including gametogenesis and planozygote formation. A recent study by Wyatt and Zingone (2013) has highlighted the importance of paying closer attention to life history phases in order to assist the predictive ability of harmful algal bloom (HAB) forming species such as *Alexandrium*. Although the sexual reproductive stages of the genus have been successfully identified within laboratory conditions (Garcés et al., 1998, Figueroa et al., 2005, Figueroa et al., 2007), the observed results may not represent cell behaviour within the natural environment. In order to achieve a greater understanding of dinoflagellate bloom dynamics and the processes influencing cell abundance, individual life stages of a species should be investigated within the natural habitat.

Cork Harbour, located on the southwest coast of Ireland is the only area along the Irish coastline to contain a resident population of the toxin producing *Alexandrium minutum*. This population blooms annually, causing frequent contamination of bivalves with paralytic shellfish poisoning (PSP) (Moran et al., 2006; Cosgrove et al., 2014). Although the life span of an *Alexandrium* bloom is generally short (Wyatt and Jenkinson 1997), periods of exponential vegetative growth within the North Channel area of Cork Harbour have been observed to exceed densities of $10^5$ cells L$^{-1}$ (Ní Rathaille and Raine 2011, Cosgrove et al., 2014). The *A. minutum* bloom will generally collapse within days of reaching its peak, and although biological parameters may play an influential role, the termination of the bloom has been often linked to changing hydrodynamic and meteorological conditions (Touzet et al., 2009). Balch (1986) discusses the influence of spring and neap tides on bloom dynamics and highlights the importance of neap tide periods for dinoflagellate cell growth.
Stratification of the water column during neap tides is thought to be essential for *Alexandrium* bloom development (Berdalet and Estrada 2005), as turbulent conditions can both deteriorate cells and induce behavioural changes (Smayda 1997). Increased tidal movement which results in cell dispersion exceeding cell growth (Okubo 1978) is most likely a common cause of bloom collapse. Other environmental factors believed to influence bloom termination include wind advection (Raine and McMahon 1998; Yamamoto *et al.*, 2002) and decreasing day length (Yentsch and Mague 1980). While the main biological factors reported to influence cell abundance include the prevalence of viral infections (Suttle *et al.*, 1990; Nagasaki 1994), parasitic infections (Chambouvet *et al.*, 2008; Montagnes *et al.*, 2008), bacterial infections (Kodama *et al.*, 2006; Su *et al.*, 2011) and grazing (Watras *et al.*, 1985; Calbet *et al.*, 2003).

A lot remains unknown about which, if any, environmental or biological stresses are responsible for inducing sexual reproduction in *Alexandrium* cells. Wyatt and Jenkinson (1997) suggest a cell abundance threshold is required before gametogenesis begins, as a reduced nearest neighbour distance is essential for gametes to successfully encounter and fuse. Although some studies have reported dinoflagellate gamete cells to be smaller than the vegetative cell (Von Stosch 1973; Kremp and Heiskanen 1999), a study by Figueroa *et al.* (2007) found virtually no difference between the size of an *A. minutum* vegetative and gamete cell. The inability to distinguish between these life stages based on morphological differences makes the task of identifying the initial onset of sexual reproduction considerably difficult. *Alexandrium* planozygote cells, resulting from the fusion of gametes, have a dark, granular appearance (Genovesi *et al.*, 2009) and are larger than either the vegetative or gamete phases (Figueroa *et al.*, 2007), making their identification possible by morphological analysis. Following the successful collision of gametes, planozygotes are reported to form within two days (Probert 1999), and can potentially stay motile for up to one week before encystment (Anderson 1998). Figueroa *et al.* (2007) observed cyst formation within an *A. minutum* culture six days after inducing sexual reproduction. Such a fast transition rate suggests the possibility of life cycle stages playing a role in bloom demise. It has also been suggested that a substantial proportion of planozygotes do not progress to encystment (Anderson *et al.*, 1985). The data presented in Cosgrove *et al.* (2014) and Garcés *et al.* (2004) show that the
amount of hypnozygote cysts which reach the sediment represents on average 2.5% of
the vegetative population based on samples collected using sediment traps.

The prediction of the timing and duration of HABs within areas of shellfish
aquaculture is highly desirable both for the protection of public health and the
alleviation of economic loss. It is necessary to understand the level of influence
discrete environmental parameters hold over the population dynamics of HAB species
before we can predict their initiation and rates of growth and mortality. Since the first
mathematical models examining the seasonal cycle of phytoplankton (Riley 1946),
model simulations have become an extremely valuable tool in the management of
phytoplankton ecology and often provide a simplified picture of a complex system. If
designed correctly, they can confirm a hypothesis already determined by
observational data and also test the viability of new hypotheses (Lehman et al., 1975).
In addition, their application can reveal gaps within the current knowledge, leading to
new areas of research (Jorgensen and Bendoricchio 2001). A coupled physical-
biological model designed by Stock et al. (2007) successfully simulates the general
timing and magnitude of an *Alexandrium fundyense* bloom in the Gulf of Maine by
combining the essential parameters of irradiance, temperature and salinity with a rate
of mortality and nitrogen dependence. The initiation of *Alexandrium* blooms in the
North Channel of Cork Harbour has also been successfully modelled using the
balance between tidal dilution and the in situ growth rate derived from light and/or
temperature control (Ní Rathaille 2007).

In this study, we investigate the role of life cycle transitions in the decline of *A.
minutum* blooms in the North Channel area of Cork Harbour, paying particular
attention to the sexual reproductive cycle. We focus on the summer periods of 2004
and 2011, years when intense bloom concentrations (>100,000 cells L\(^{-1}\)) developed. A
simple box model is used to simulate the life stages of vegetative cell growth,
gametogenesis, planozygote formation and encystment using the environmental
parameters of irradiance, temperature and tidal dilution. Hindcasting the model
simulation to the cell densities observed in 2004 and 2011 allowed us to investigate
the accuracy of the model. Varying rates of gamete formation and gamete fusion,
along with the length of days required for a planozygote cell to encyst were tested in
the simulation to find the best-fit output.
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METHODS

Study area

The North Channel is a region of Cork Harbour (figure 1B) 9km in length and between 0.5 and 1 km in width with a tidal excursion of 6-8 km. A 200 m opening on the south eastern shore connects the channel to the main harbour via a shallow passage (5-10 m deep) at east Ferry which is ~2 km in length. The average tidal range in the channel (measured at Cobh) is 3.7 m on a spring tide and 2 m on a neap tide. Extensive mudflats become exposed in the western end of the channel, which on a high tide has an average water depth of 3 m. Fine sediment and retentive properties in the western section of in North Channel make it a suitable environment to accommodate the initiation and development of *A. minutum* blooms (Ní Rathaille and Raine 2011). The samples were collected at five stations (R, Q, P, O, NO) along a transect across the channel with sampling intervals of ~0.5 km. Environmental data and water samples were collected weekly in the North Channel area of Cork Harbour (figure 1A) between May 25th and July 13th 2011, and May 26th and June 30th in 2004. Instruments measuring levels of irradiance and water temperature were also placed at locations in the channel all year round.

Figure 1. The Study Area. A) Bathymetric map of Cork Harbour, Ireland, highlighting the North Channel located in the northeastern half of the Harbour. B) Map of the North Channel showing the five water sampling stations occupied on each field survey. The location of the moored temperature sensor is also indicated.
Environmental data

Irradiance was measured throughout both 2004 and 2011 using a Licor LI190 quantum sensor. This was mounted 3 m above ground level in a shade free environment on the northern shore of the North Channel and data were logged hourly with a LI1000 data logger. Missing data periods were supplemented by the global solar radiation measured using a pyranometer operated by Met Eireann at the weather station situated at Valentia observatory, Co. Kerry (51°54’N; 10°21’W). Before applying to the model, both data sets of irradiance measurements were converted to mean hourly water column photosynthetically active radiance (PAR), based on a 14 hour day. Mean global irradiance ($\Sigma G$) recorded in Joules m$^{-2}$ s$^{-2}$ were converted into $\mu$E·m$^{-2}$·s$^{-2}$ (where 1 $\mu$E·m$^{-2}$·s$^{-2}$ is equal to 1 $\mu$M·m$^{-2}$·s$^{-2}$ of photon). The following equation was used for the conversion:

$$I = \frac{\Sigma G}{14} \times 2.777 \times \frac{27.7}{6.022}$$

$\Sigma G$ was converted to W·m$^{-2}$ using the factor 2.778 before being converted to quanta·m$^{-2}$·s$^{-1}$ using the factor 27.7 (Kirk 1983), and finally to moles using Avogadro’s number. The value of $I$ was converted to the level of irradiance available in the water column ($I_0$) by multiplying by 0.225, which accounts for a loss of irradiance below the water surface. Finally, irradiance at depth ($I_z$) was calculated using the following equation, where $\lambda$ is the vertical light attenuation coefficient ($\text{m}^{-2}$) (see appendix I) and $h$ is the average water depth. See Ní Rathaille 2007 for more detail.

$$I_z = I_0 / (2^* h) * (1 - \exp(-\lambda * h))$$

Temperature data were also logged hourly in the channel using a TidbiT sensor moored just above the sea bed at a location between stations O and P (figure 1B). The average daily temperature was used in the development of the model. Tidal dilution for the North Channel was calculated using equation 1a derived from the use of a hydrodynamical model of Cork Harbour (Ní Rathaille 2007; Ní Rathaille et al., 2009) where tidal range (R) was calculated from the Cork Harbour 2004 and 2011 tide tables (measured at Cobh). At each sampling station in 2011, salinity and temperature...
measurements were recorded at 1 m intervals from the surface to the sea bed using a WTW T/S probe. In 2011, chlorophyll levels were determined weekly at each station: One litre of seawater was filtered onto a GF/C Whatman filter and stored at -20 °C until ready for analysis using the fluorometric method outlined in Tett (1987).

\[ D = 0.0362 \cdot \exp(0.6842\cdot R) \]  \hspace{1cm} (1a)

**Cell density**

Surface and sub-surface water samples for cell enumeration were retrieved weekly from all five stations using a bucket and 5L Niskin bottle respectively. The water samples were initially filtered through a 150 µm mesh before further processing to eliminate larger particles. For light microscopy analysis, sub-samples were stored in 50 mL cell culture bottles and preserved with 0.4 mL Lugol’s iodine. The samples were then kept in the dark at room temperature until *A. minutum* cell densities were calculated using inverted microscopy (McDermott and Raine 2010). For fluorescent in situ hybridisation (FISH) analysis, 2 litres of water (pre-filtered through a 150 µm mesh) were collected onto a 5 µm nylon mesh filter 47 mm in diameter. The collected material was then backwashed using 0.22 µm filtered seawater (FSW), into a 50 mL polypropylene centrifuge tube and fixed with formalin (1% final concentration v/v). The samples were kept at 4 °C in the dark for a maximum of six hours. In the laboratory, the preserved samples were centrifuged to form a pellet at 4000g for ten minutes before re-suspension in FSW. This process was carried out three times to ensure the removal of residual formalin which can affect the quality of microscopy analysis. The washed pellet was finally resuspended in 14 mL ice-cold methanol and stored at -20 °C until further processing. The *A. minutum* probe MinA with the fluorochrome CY-3 (Touzet *et al.*, 2008) was hybridized to the samples prepared for FISH using the method as described by Miller and Scholin (1998). The probe was hybridized to an aliquot (0.5 - 1 mL) of the methanol suspension; the volume depended on the concentration of cells within the sample (as determined from light microscopy analysis). The hybridized sample was filtered onto a 1.2 µm polycarbonate filter 25 mm in diameter. Following a wash buffer rinse, 5 µL of Calcofluor (100 µg mL⁻¹) was added to the filter and it was placed on a glass slide and finally secured with a cover slip. *A. minutum* cells were identified and enumerated
using epifluorescence microscopy and appropriate CY-3 optical filter set described in Touzet and Raine (2007).

**Life-stage analysis**

In addition to the quantification of *A. minutum* cells in their vegetative state, an attempt was made to identify and enumerate planozygote cells in the water column in 2011. Following a settling period, the Lugol’s preserved samples were analysed by light microscopy and based on specific criteria in their morphology, planozygote cell concentrations were calculated in the weekly samples. The relative proportion of observed planozygote cells to *A. minutum* planktonic cells was then determined. The morphological characteristics of a planozygote cell include a larger size than the vegetative cell and darker in colour with a more granulated appearance. Based on the assumption planozygotes encyst within seven days, an estimate of the percentage of planozygotes cells which progressed to encystment (EN) was also made. This was estimated using equation 2a, where N represents total observed planozygote cells L\(^{-1}\) (excluding the final week of sampling), C represents the total density of newly formed cysts cm\(^{-2}\) measured in the sediment traps (beginning seven days after planozygote cells appeared and for the consecutive weeks following) and h the average water column height. This calculation was carried out once planozygotes had first appeared in the water column (four weeks after sampling commenced). This equation also accounts for differing units, as cysts are expressed in cm\(^{-2}\) and cells presented in L\(^{-1}\).

\[
EN = \frac{C \times 10000}{(N \times h) \times 1000} \times 100 \quad (2a)
\]

In order to verify the features used to identify planozygote cells, experiments were carried out in parallel using three strains of *A. minutum* cultures, all originating from the North Channel. 20 mL of ½ IMR medium (Eppley *et al.*, 1967), 20 mL of ½ IMR medium without phosphate added and 20 mL of ½ IMR medium without nitrate added were added separately to 50 mL cell culture flasks and inoculated with exponentially growing *A. minutum* cells to give a final concentration of ~500 cells mL\(^{-1}\). Each flask was observed every 2 days for 14 days for signs of sexual reproduction. Following the induction of sexual reproduction, 1 mL aliquots were extracted from the sample and
preserved with Lugol’s iodine as mentioned above. This enabled the comparability between the morphology of the planozygotes cells observed in culture and within the natural samples in 2011.

Model development

An *A. minutum* bloom termination model controlled by the onset of sexual reproduction was designed to test a range of gamete formation and gamete collision rates which best simulated the observed cell densities in 2004 and 2011. No reversion factor was included for gamete cells. It was assumed that once they underwent gameteogenesis they remained part of the planktonic population until successfully fusing to form a planozygote cell, with tidal dilution as the only other factor affecting their density. In order to simulate bloom development, a net growth rate (day^{-1}) for *A. minutum* was determined by variations in irradiance, temperature and tidal dilution. The relationships between growth rate and both irradiance and temperature, as well as that between tidal range and tidal dilution in the North Channel of Cork Harbour, were those derived by Ní Rathaille (2007). In essence, the model is based on the premise that for blooms to initiate and develop, the growth rate must exceed the tidal dilution rate.

Temperature ceases to affect growth rate above 15 °C, and in situ irradiance (PAR) ceases to have an effect above water column average irradiance of 100 µE ·m^{-2}·s^{-1}. Neither variable had a negative/inhibitory effect on growth in the in situ ranges of these parameters found in the North Channel. A maximum growth rate of 0.54 day^{-1} (µ_{max}) was implemented (Ní Rathaille and Raine 2009).

The model was run with a time step of one day, using the following equations:

\[
\mu (I) = \mu_{max} \frac{(Iz/100)}{ \mu_{max} } \text{ when } I < 100 \text{ µE ·m}^{-2}·s^{-1} \quad (3a)
\]

\[
\mu (I) = \mu_{max} \text{ when } I > 100 \text{ µE ·m}^{-2}·s^{-1} \]

\[
\mu(T) = \mu_{max} \cdot (0.1 \cdot T - 0.5) \text{ when } T < 15 \quad (3b)
\]

\[
\mu(T) = \mu_{max} \text{ when } T \geq 15
\]

\[
\mu = \mu (I,T) \quad (4)
\]
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The effects of irradiance and temperature were multiplicative. So, for example, if the temperature is <15 °C and the water column irradiance < 100 µE · m⁻² · s⁻¹, then the growth rate (µ) would become

$$\mu = \mu_{\text{max}} \times (0.1 \times T - 0.5) \times (I_z / 100)$$

(5)

The net increase in biomass (µₙₑₜ) becomes the balance between growth and tidal dilution (D):

$$\mu_{\text{net}} = \mu - D$$

(6)

Tidal dilution was estimated from tidal range using the equations defined in Ní Rathaille (2007)

A rate of gamete formation and gamete fusion was introduced to simulate bloom termination. The timing of the introduction was based on the observed initiation of sexual reproduction. Newly formed gametes were separated from the vegetative population, yet remained part of the visible planktonic biomass before successful fusion. Planozygote cells were formed 24 hours after gamete fusion and remained in the water column up to seven days before encystment. The visible biomass consisted of the total vegetative cells, total gametes and total planozygotes on any given day.

The effect of tidal dilution was applied to each life stage. A variance study was carried out to find the best-fit output from a range (10-90%) of gamete formation and gamete fusion rates. This was achieved by creating a Sum of Squared Errors (SSE) matrix using observed and simulated cell density values. The lowest SSE indicated the best-fit value for the simulation. Using both observed and simulated cyst density values from 2011, the best-fit length of days from planozygotes formation to encystment was also calculated.

The model simulations for both 2004 and 2011 were also used to calculate the proportion of planozygotes in the A. minutum planktonic biomass, the proportion of A. minutum planozygote cells which underwent successful encystment; calculated as the fraction of cysts L⁻¹ which formed from the vegetative biomass five days earlier, and the proportion of the vegetative population which successfully encysted.
RESULTS

Environmental parameters

Figures 2 and 3 illustrate the mean incident global irradiance and mean hourly PAR in 2004 and 2011 respectively. An irradiance level of 100 µE·m⁻²·s⁻¹, suggested as the minimal level for optimum *A. minutum* growth (Ni Rathaille *et al*, 2009), was exceeded during the period of mid-March to mid-September. Figures 4A and 4B present the mean daily water temperature in the North Channel for 2004 and 2011 respectively. The gap in the data between the end of November and mid December is due to battery failure in the TidbiT sensor. Water temperatures ranging between 15°C and 20°C are thought to favour the growth of *A. minutum* cells (Ni Rathaille *et al*, 2009) and were observed generally between the months of May to September. A four month window of favourable environmental parameters is therefore presented in this period for the optimum growth of *A. minutum* cells. Figure 4C shows the average temperature (°C) while 4D illustrates the salinity (PSU) measurements at 0 m from each sampling station over the eight week period in 2011. The highest and most consistent temperatures above 15 °C are evident in the last three weeks from June 29th to July 13th, reaching up to 18°C. There is little variance in the salinity levels over the entire sampling period.

![Figure 2. Daily global solar radiation measured at Valentia, Co. Kerry for 2004. The primary y-axis indicates mean hourly underwater irradiance levels for a 2 metre water column calculated from the solarimeter data (see text).](image-url)
Figure 3. Daily global solar radiation measured at Valentia, Co. Kerry for 2011. The primary y-axis indicates mean hourly underwater irradiance levels for a 2 metre water column calculated from the solarimeter data (see text).

Figure 4. Mean daily temperature measured in the North Channel using the TidbiT sensor (see figure 1B for location) for 2004 (A) and 2011 (B), weekly average sea surface temperature (C) and weekly average salinity (D) averaged across the North Channel from station NO-R in 2011.
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Observed bloom dynamics
The average cell densities recorded across the channel in 2004 and 2011 were 620,000 cells L\(^{-1}\) and 830,000 cells L\(^{-1}\) respectively during bloom peak, falling to < 10,000 cells L\(^{-1}\) within 14 days (Figure 5). Using the growth function described above, the initiation and development of both blooms could be linked to the cycles in tidal dilution. This is because treating growth as a function of temperature and irradiance alone, blooms take approximately 10-14 days to develop with the water column irradiance levels typical of the North Channel (Ni Rathaille, 2007). Populations therefore need to survive through a spring tide, as in 2004 when development occurred through a low (equinoctial) spring tide in June, or else bloom through a neap tide when irradiance levels are above average, as occurred in July 2011. The second spring tide in June is generally particularly low due to the summer solstice. During the summer solstice period around June 21st, the ocean's tides are at their lowest ebb of the year, resulting in reduced spring tidal ranges. Before the bloom events, tidal dilution was too great to allow for significant population growth. Although the duration of the blooms are often relatively short (~14 days), *A. minutum* often becomes the dominating dinoflagellate during intense bloom periods. In 2011 the *A. minutum* population comprised >90% of the dinoflagellate community.

![Figure 5. Average *A. minutum* cell densities cells L\(^{-1}\) across the North Channel in 2004 and 2011, highlighting the two week time difference between bloom peaks.](image-url)
during the height of its bloom, outcompeting species of *Scrippsiella*, *Akashiwo*, *Gymnodinium* and *Heterocapsa*. Throughout the eight week sampling period, it was noted that chlorophyll concentrations peaked at 9.5 µg L⁻¹ during the week of maximum recorded *A. minutum* cell densities.

**Planozygote cell features**

In 2011, *A. minutum* planozygote cells began appearing in the water column on June 22nd (week five of sampling), 14 days before maximum vegetative cell intensity was observed. The cells were identified as planozygotes based on their dark, granular appearance and larger size. On average, the planozygote cells measured ~25% larger than *A. minutum* vegetative cells (see figure 6). The length of a typical planozygote cell was 22-25 µm, with a transdiameter of ~20 µm, while the length of a vegetative cell ranged typically from 19-21 µm with a transdiameter of 15-17 µm. Both cell types were identified as *A. minutum* using Calcofluor staining (see figures 7A-D). The first apical and sixth precingular plates are of a typical structure for an *A. minutum* cell, with the sixth precingular plate being more high than broad. The cross culture experimentation indicated signs of sexual reproduction in *A. minutum* cells by producing larger darker cells which were also on average 25% larger than the vegetative cell (figures 8A, B). Double flagella were not visible on the presumed planozygotes cells in either the field or cell culture samples, most likely due to the shedding of the flagella following preservation. Figure 9A shows a Calcofluor stained mature planozygote cell shedding its theca under epifluorescence microscopy (illustrated by a white arrow). Figure 9B shows the same planozygote cell hybridized with an *A.minutum* specific oligonucleotide probe, exposing its granular appearance (illustrated by a white arrow).
Figure 7. *A. minutum* cells isolated from the North Channel. Figures A and B illustrate a planozygote cell with Calcofluor staining (A) and under light microscopy (B). The first apical plate and sixth precingular plate are highlighted to confirm its identity as *A. minutum*. A vegetative cell is illustrated with Calcofluor staining (C) and light microscopy (D) highlighting similar morphological characteristics.

Figure 8. Micrograph of *A. minutum* planozygote formed in culture from cross experimentation (A) and micrograph of *A. minutum* vegetative cell in culture (B). Note the darker and larger features of the planozygote cell.

Figure 9. Micrographs (same image) taken with epifluorescence microscopy using Calcofluor staining (A) and *A. minutum* FISH probes (B). The images show a planozygote cell (center) shedding its theca. The larger, more granulated appearance of the planozygote cell is also demonstrated.
Chapter V

Proportion of *A. minutum* population involved in sexual cycle

Table 1 illustrates the proportion of the *A. minutum* planktonic population comprised of planozygote cells during weeks 5-8 of sampling. The enumeration of Lugol’s preserved samples found that planozygotes cells composed 14% of the planktonic *A. minutum* population one week prior to the bloom, 9% during the week of peak cell density and 12% one week after. The highest percentage of planozygotes cells (39%) was observed two weeks after the bloom peak. Thus, the average proportion of motile planozygote cells observed within the overall planktonic population was 18%. The model simulation for 2011 estimated the average proportion of planozygote cells within the total planktonic population during the same period of June 22nd to July 13th and found it to be 13%. In 2004, the simulation estimated an average of 21% of the *A. minutum* planktonic population was composed of planozygote cells between June 14th and June 30th.

Table 1. Observed *A. minutum* vegetative and planozygote cells L^{-1}, resting cysts cm^{-2} as measured by sediment traps, and the proportion of both observed and model simulated planozygote cells L^{-1} in the overall *A. minutum* planktonic population during the last four weeks of sampling in 2011.

<table>
<thead>
<tr>
<th>Date</th>
<th><em>A. min</em> vegetative cells L^{-1}</th>
<th><em>A. min</em> planozygote cells L^{-1}</th>
<th><em>A. min</em> resting cysts cm^{-2}</th>
<th>Observed % planozygote cells L^{-1}</th>
<th>Simulated % planozygote cells L^{-1}</th>
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<td>7/13/2011</td>
<td>7000</td>
<td>2730</td>
<td>1327</td>
<td>39</td>
<td>22</td>
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</table>

The cumulative number of planozygotes observed during the sampling period (excluding the final week) was 117,370 cells L^{-1}, while the total cyst input to the sediment during weeks 6, 7 and 8 was 5200 cm^{-2} (table 1). See Cosgrove *et al.* (2014) for cyst input calculation. Using an average water height of 4 m, it was estimated that the fraction of observed planozygotes which progressed to form hypnozygotes was 11%. The model simulation for 2011 estimated the percentage of newly formed planozygotes which progressed to encystment was 18%.
Finally, the model simulations estimated the average percentage of *A. minutum* vegetative cells which successfully encysted as 3.1% and 3.6% in 2004 and 2011 respectively. Refer to Cosgrove *et al.* (2014) for observed encystment rates in the years 2004 and 2011.

**Model simulation: 2011**

![Figure 10](image)

Figure 10. Model simulation of the initiation and termination of the 2011 *A. minutum* bloom plotted with observed cell densities L⁻¹ and tidal dilution

Figure 10 shows the model simulation for the termination of an *A. minutum* bloom in 2011 as a result of sexual reproduction. Tidal dilution and the observed cell densities over the summer period are also shown. The timing and length of the simulated bloom is comparable to the observed, developing towards the end of June and collapsing within 21 days. The maximum growth rate (0.54 day⁻¹) is sustained intermittently from the beginning of May to mid August, as both irradiance and temperature levels generally exceeded 100 µE m⁻²·s⁻¹ and 15 °C respectively. A tidal dilution factor regulated the net growth rate, allowing it to reach up to 0.36 day⁻¹ between the 22nd and 29th of June, as a result of the low summer spring tide. Based on the observed timing of planozygote cell formation, *A. minutum* gamete formation was introduced to the simulation from June 22nd. Table 2 shows a matrix of Sum of Squared Errors (SSE) generated in order to find the best-fit output for both the rate of gamete formation and gamete fusion. Figure 11A illustrates the trend in SSE, as values for gamete formation were varied between 10-90% and the value for gamete fusion was held at 10%. The curve reaches its lowest point at a rate of 40%, indicating the best-fit
simulation value. In figure 11B, the rate of gamete formation is held at 40% while varying (10-90%) gamete fusion rates were plotted to find the lowest SSE. Increasing the rate of gamete fusion, decreased the cell density output and generated a greater difference between the observed and simulated data. This resulted in the lowest value of gamete fusion (10%) producing the lowest SSE.

Table 2. Matrix of sum of squared errors (x10^{10}) generated for the 2011 model using a range of gamete formation rates and gamete fusion rates. The values that best fit the data gave the smallest sum of squared errors (underlined)

<table>
<thead>
<tr>
<th>Successful collision (%)</th>
<th>10</th>
<th>20</th>
<th>30</th>
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Figure 11. Sum of squared of errors (x10^{10}) between cell numbers observed during A. minutum bloom in 2011 and simulated cell numbers as (A) gamete formation was varied at a successful fusion rate of 10% and (B) gamete fusion rates varied at a gamete formation rate of 40%. Data for observations are given in table 2.

Table 3 shows a matrix of SSE determining the best-fit value for the length of days required for a planozygote cell to encyst, using a range of gamete fusion values and a
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gamete formation rate of 40%. The time length ranged from 1-7 days, while the values of gamete fusion, as above, ranged from 10-90%. Figure 12A shows the effect of a varying time length for the encystment of a planozygote while gamete fusion was held at 20%. The curve attains a minimum value after five days. Figure 12B shows the SSE values for gamete fusion rates between 10 and 90%, while the length of time before encystment is held at five days. In this case, the lowest SSE was found when the gamete fusion rate was 20%.

Table 3. Matrix of sum of squared errors (x10^6) generated for the 2011 model using a range time periods (days) from planozygote formation to encystment and gamete formation rates. The values that best fit the data gave the smallest sum of squared errors (underlined).

<table>
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Figure 12. Sum of squared errors (x10^6) between cell numbers observed during *A. minutum* bloom in 2011 and simulated cell numbers as (A) no. days before encystment was varied at a successful fusion rate of 20% (B) successful fusion rates varied at a time period of 5 days. Data for observations are given in table 3.
Figure 13 presents a simulation of *A. minutum* bloom intensity and length for 2011, based only on the environmental parameters of irradiance, temperature and tidal dilution. The bloom initiates towards the end of April, peaking at a cell density of $>10^{13} \text{ cells L}^{-1}$ by October and gradually reducing into the winter months. By December cell densities still exceed $10^{14} \text{ cells L}^{-1}$.

**Model simulation: 2004**

A simulation of the observed *A. minutum* bloom in 2004 is shown in figure 14, showing little discrepancy against the observed cell densities between May 26th and June 30th 2004. As in 2011, the main environmental parameters influencing growth are irradiance and temperature, both frequently reaching optimum levels between mid-May and early September. As no observed data for the initiation of sexual reproduction is available for 2004, gamete formation was introduced to the simulation during the week preceding the observed bloom peak. Reduced tidal dilution allowed
the net growth rate day\(^{-1}\) to reach 0.35 day\(^{-1}\) during the first week of June. Table 4 shows the matrix of SSE values calculated to determine the best-fit rate of gamete formation and gamete fusion ranging from 10-90%. Figure 15A shows the result of varying gamete formation rates, with gamete fusion held at a value of 20%. The lowest point of the curve is evident at a rate of 40%, producing the same optimum rate as the 2011 simulation. The SSE trend for varying gamete fusion rates are shown in figure 15B, with the lowest value achieved at a rate of 20%.

Table 4. Matrix of sum of squared errors (x10\(^{10}\)) generated for the 2004 model using a range of gamete formation rates and gamete fusion rates. The values that best fit the data gave the smallest sum of squared errors (underlined)

<table>
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<th>Successful collision (%)</th>
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</table>

Figure 15. Sum of squared of errors (x10\(^{10}\)) between cell numbers observed during *A. minutum* bloom in 2004 and simulated cell numbers as (A) gamete formation was varied at a successful fusion rate of 20% (B) gamete fusion rates varied at a gamete formation rate of 40%. Data for observations are given in table 4
DISCUSSION

The various life-history stages of an *Alexandrium* cell can often complicate bloom prediction efforts, as the timing of the transition between stages and the resulting influence on population dynamics is an area which remains largely unexplored. Laboratory experimentation has granted some insight into the factors triggering sexual reproduction (Figueroa *et al.*, 2007), yet cell behaviour observed in culture is not always an accurate representation of cell behaviour in the natural environment. For example, the reluctance of *A. minutum* planozygote cells to encyst within culture when tested under a variety of conditions has previously been demonstrated (Garcés *et al.*, 1998; Figueroa *et al.*, 2005). The principle of this study was to focus on the initiation and effect of sexuality on *A. minutum* populations in their natural environment. *A. minutum* cells, like many other dinoflagellate spp., undergo three steps to complete the route of sexual reproduction from vegetative cell to resting cyst. Gametogenesis initiates the process, followed by gamete fusion/ planozygote formation and finally encystment to form a hypnozygote cell. Various conditions have been described as the catalyst for initiating sexual reproduction, including a vegetative cell threshold (Wyatt and Jenkinson 1997; Anglès *et al.*, 2012a), decreased salinity levels, increased temperature levels (Figueroa *et al.*, 2011) and both nutrient deficiency (Probert *et al.*, 2002; Figueroa *et al.*, 2007) and nutrient repletion (Anderson 1998; Garcés *et al.*, 2004). In 2011 in the North Channel, *A. minutum* cells entered their sexual phase of reproduction during exponential growth at a cell density of $8 \times 10^4$ cells L$^{-1}$, and two weeks before the bloom reached its peak. During this period, temperatures were at their highest, irradiance exceeded 100 µE m$^{-2}$ s$^{-1}$ and little variance was observed in salinity levels. Previous studies have also observed signs of sexuality prior to maximum cell concentrations and during conditions which favor optimum growth (Anderson 1983; Kremp and Heiskanen 1999; Garcés *et al*.; 2004; Anglès *et al.*, 2012a).

Although the results of this study were generated using a simple ecological model, its application can expose fundamental questions regarding population dynamics (Murray 1989). The simulation output suggests sexuality is capable of terminating an intense bloom (>100,000 cells L$^{-1}$) of *A. minutum* within a three week period, with tidal dilution as the only other contributing factor. Bloom demise was achieved when
gamete formation reached 40% assuming the cessation of binary fission following gameteogenesis. It has been reported that both gametes and planozygotes have the ability to revert to a vegetative state, regaining the ability to asexually divide (Figueroa and Bravo 2005; Figueroa et al., 2006), but a reversion factor was not included in this simulation. The model assumed gamete cells remained part of the total planktonic population until successful fusion was achieved, and all planozygote cells which were not flushed out of the system, successfully encysted within 7 days. Such a high level of success is most likely improbable, as tidal dilution is assumed an exclusive factor suppressing cell densities. In reality, a variety of factors may inhibit cells from reaching the next stage of sexual reproduction and play a role in bloom termination, including bacterial and viral infections, parasitic activity and levels of shear or turbulence within the water column. As Alexandrium gamete cells have been described as virtually indistinguishable from vegetative cells (Nagai et al., 2003; Band-Schmidt et al., 2003; Figueroa et al., 2007), it remains to be seen as to whether a gamete formation rate of 40% is achieved in a coastal estuarine system such as the North Channel and can potentially cause the collapse of a bloom. A model simulation illustrating the impact of environmental conditions alone on A. minutum bloom produced an unrealistic representation of bloom magnitude and length. This confirms the influence of other controlling factors whether physiological or ecological on bloom dynamics in this region.

Wyatt and Jenkinson (1997) have argued that influential factors including cell dispersion, turbulence and the potential heterothallic nature of Alexandrium species should result in successful gamete fusion being a ‘rare event’. Model simulations for both 2004 and 2011 estimated that successful gamete collision rates of 20% and 10% respectively, produced the best comparison between the simulated output and field observations. In 2011, the simulation calculated the average proportion of planozygote cells within the total planktonic population to be 13% during the period of June 22nd to July 13th. As calculated from the field data, an 18% proportion of motile planozygote cells in the overall planktonic population were observed within the same period. In 2004, the estimated proportion of planozygote cells was 21%. The comparability between results suggests the model output is accurately representing the level of planozygote formation occurring in the field. Yet the reliability of comparing both data sets has to be questioned as the simulation produced
a daily output, while the observed data were recorded weekly. Increased disparity may also result from the fact observed planozygote cell densities were calculated using discrete surface water samples, accounting only for motile planozygotes and eliminating the cells in their mature stage of development. Nonetheless, the observed proportion of planozygotes (18% of the vegetative cells) suggests gamete fusion not to be a rare event within the North Channel. The presumed shedding of flagella following the addition of a preservative, made it difficult to capture an image which confirmed the presence of two flagella following gamete fusion, so the quantification of planozygote cells within field samples was based on cell morphology and size alone. But this approach has been successfully employed in previous studies (Anderson 1980, Genovesi et al., 2009, Anglès et al., 2012b), all of which described a planozygote cell as larger and darker relative to the vegetative cell. The size of the planozygote and vegetative cells imaged in the 2011 field samples are believed to be smaller than the equivalent live cells due to shrinkage as a result of the preservative Lugol’s iodine (Menden-Deuer et al., 2001)

In 2011, the parameters generating the most accurate simulation of the observed cyst concentration; a gamete fusion rate of 20% and encystment after five days produced a total output of 7200 *A. minutum* cysts cm\(^{-2}\) during the period of June 30\(^{th}\) and July 13\(^{th}\). Sediment traps deployed in the North Channel in 2011 calculated a total density of 5200 *A. minutum* cysts cm\(^{-2}\) produced during the same time period in 2011 (Cosgrove et al., 2014), installing confidence in the accuracy of the simulation. In addition, the average proportion of observed *A. minutum* vegetative cells which underwent successful encystment as measured during four summer periods in the North Channel (including 2011) averaged at 2.5%, while the model simulations estimated the average proportion of the vegetative cell biomass to encyst was 3.6% and 3.1% in 2004 and 2011 respectively. Such comparable results suggest a degree of confidence should be installed in a gamete fusion rate of 20%. It is likely the length of days between planozygote formation and encystment will vary within the natural environment, with factors such as cell intensity previously shown to alter encystment behaviour (Uchida 2001). But the fact the encystment % remains within a range of 2-3.2% when the time period is modified from one day to seven, yet extends from 0.1 to >100% when the gamete fusion rate ranges from 10-90%, suggests the model is less sensitive to length of days than encystment %. One such factor shown to affect
encystment is the complex heterothallic mating behaviour of *A. minutum* cells (Figueroa *et al.*, 2007). Heterothallic behaviour requires more than two types of sexual types (+/-) for sexual reproduction and so may curtail the process of gamete fusion due to an imbalance in the ratio of mating types. This may be a contributing factor as to why only an estimated 1 out of 5 gametes successfully collide, and the consistently low cyst production observed within the North Channel.

**CONCLUSION**

This study shows that sexual reproduction has the potential to contribute substantially to bloom termination. It illustrates the onset of sexual reproduction 7 days prior to maximum cell concentrations and, more notably, the ability of the population to achieve a bloom density of $>8 \times 10^5$ cells L$^{-1}$ after sexuality has begun and when a proportion of cells are presumed to be no longer contributing to bloom development. When gamete formation reaches 40% day$^{-1}$, it appears to impede bloom growth. Due to the influence life-history stages exert over bloom dynamics, knowledge surrounding each stage of sexual reproduction is essential to be able to better understand and predict their timing and magnitude. The application of a simple ecological simulation suggests that although successful encystment percentages are low, earlier stages of this life history phase may play a more crucial role in *A. minutum* population dynamics than once previously thought.

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Chapter V


Chapter VI

Interannual variability in *Alexandrium* spp. cyst densities in Cork Harbour, Ireland and their relation to bloom intensity.

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Chapter VI

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ABSTRACT

The distribution of *Alexandrium* cysts in the sediments of Cork Harbour, Ireland, have been investigated since 2003 in order to better understand the dynamics of *Alexandrium* spp. in this region. Cyst densities of *A. minutum* and *A. tamarense* were consistently higher in the North Channel than elsewhere in the Harbour, and it is this region where blooms of toxin producing *Alexandrium* initiate. During each survey, both horizontal and vertical distributions of cysts were investigated during the winter months. The means and variance of the horizontal cyst distribution in the top 1 cm of sediment in the North Channel were compared between years, and the data were also analysed in conjunction with the maximum observed vegetative cell densities in subsequent summers. The variability in the cyst distribution is discussed in relation to the hydrodynamics of the North Channel; in particular the coincidence of the highest cyst densities with the location of tidally induced eddies. Nevertheless, the results show a statistically significant decreasing trend in cyst density since 2003. An analogous decreasing trend in the maximum observed bloom cell density was also apparent since 2004 when an exceptional *Alexandrium* bloom (5*10^5 cells L^-1) occurred. The potential of the winter cyst density in controlling the intensity of summer blooms is discussed.

**Keywords:** *Alexandrium*; cyst distribution; hydrodynamics; Cork Harbour;
INTRODUCTION

Shellfish aquaculture is a significant component of the economy of the southwest coast of Ireland (Parsons 2005). The occurrence of harmful algal blooms has hindered the development of the industry, in particular through contamination of shellfish with algal biotoxins (McMahon et al., 1998). Cork Harbour, Ireland’s most industrialised harbour located on the southern Irish coast has a history of episodic contamination of shellfish with paralytic shellfish toxins, and blooms of *Alexandrium* spp. can occasionally reach high cell density (ca. 10^3 m L^{-1}; Ni Rathaille 2007). The first documented outbreak occurred in 1987, and subsequently there have been frequent bans on shellfish harvesting in the area. Notable exceptions have been the years 1999 and 2001. Toxin contamination most frequently occurs between mid-June and mid-July. Cork Harbour is the only site in the Republic of Ireland where paralytic shellfish poisoning contamination has occurred. There are at least thirty recognised *Alexandrium* species (Balech 1985, 1989). To date, *A. minutum*, *A. tamarense* and *A. ostenfeldii* have been identified in Cork Harbour (Touzet et al., 2008). It was originally thought that *A. tamarense* was the causative organism of these toxic events. However, toxin profiles of cultures isolated from the estuary have indicated that the causative organism is *A. minutum* (Touzet et al., 2007). Isolates had a toxin profile of the two potent gonyautoxins GTX2 and GTX3 which have been characteristic of contaminated stock (Furey et al., 1998; Touzet et al., 2007).

The life cycle of *Alexandrium* has a resting cyst stage, in which state it can overwinter or stay dormant for several years in adverse conditions (Anderson et al., 1987). Excystment into the planktonic vegetative form initiates *Alexandrium* blooms. Knowledge of the distribution and cyst density is therefore potentially vital information if the effects of *Alexandrium* blooms are to be managed and mitigated. This study examined the variation in the distribution and density of *Alexandrium* cysts in Cork Harbour since 2003.
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METHODS

Cyst surveys were carried out in the north-eastern section of Cork Harbour known as the North Channel (figure 1). It is in this part of the estuary, which has dimensions 8-9 km by 0.5 km, where Alexandrium blooms initiate and where the highest dinoflagellate cyst densities can be found (Ni Rathaille, 2007). Sub-tidal sediment samples were obtained using a manually operated Ekman Birge bottom sampler (HydroBios, Kiel). Triplicate sub-samples of surface sediment were taken from this using sawn off 50 mL syringes that were filled to the depth of the sediment in the sampler. Intertidal samples were collected from the shoreline manually, also using 50 mL core syringes. The sediment was kept in the dark at 4º C until further analysis in the laboratory. In order to quantify the number of cysts in the sediment, the surface 1 cm of each sediment core was removed, mixed to ensure homogeneity, and from this a measured, weighed volume (usually 0.4 mL) was put into a beaker of water, ultrasonicated, and the cysts were extracted using density gradient centrifugation with sodium polytungststate. Prior to 2007, Ludox was used for the density gradient. The number of Alexandrium cysts was counted using a Sedgwick-Rafter cell at x100 magnification.

Cyst surveys were carried out between mid-September and mid-April when Alexandrium can be expected to be in the dormant phase (Table 1).

RESULTS

The survey carried out on 6-7 April 2010 involved thirteen sampling sites, three of which were intertidal (figure 1). Cyst densities of A. tamarense ranged from 5-20 cysts · g dry wt sediment⁻¹. Cyst densities of A. minutum were more variable and generally higher in the range of 5-80 cysts · g dry wt sediment⁻¹. Higher cyst densities were found in the western (inner) end of the North Channel as illustrated in figure 1. Mean cyst densities of A. tamarense and A. minutum were 11 and 38 cysts g⁻¹ respectively. These data can be compared with those obtained during the dormant season in previous years (Table 1). A decline in A. tamarense and A. minutum cyst densities is evident in years proceeding 2003.
Results from the annual surveys since 2003 identified six suspected hotspots retaining increased cyst densities during the annual dormant season (figure 2). As indicated on the map, a location far west in the channel, a location far east and the region surrounding a lagoon site were found to have retentive sedimentary properties which grant opportunity for cyst settlement. Seedbeds of considerable density were also found in three regions near the mouth of the channel, where gyre formations are known to occur. These gyre formations have been visually identified on the water surface during annual surveys. If we are to regard cysts as passive particles within the water column it becomes clear as to how these distinctive water movements can influence the distribution of the cysts within the sediment.

Figure 3 illustrates *Alexandrium* resting cyst densities against *Alexandrium* vegetative cell densities from the years 2003-2010. A drop in both *A. minutum* and *A. tamarense* cyst densities coincided with a sudden decline in the vegetative cell densities after the winter period of 2003-2004.
Table 1. *A. tamarense* and *A. minutum* cyst density data, the North Channel (2003-2010).

<table>
<thead>
<tr>
<th>Dormant Season</th>
<th>Date Sampled</th>
<th><em>A. tamarense</em> (cysts per gm dry sediment)</th>
<th><em>A. minutum</em> (cysts per gm dry sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>max</td>
<td>mean</td>
</tr>
<tr>
<td></td>
<td>9-12 Mar 2004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2004/2005</td>
<td>21 Oct 2004</td>
<td>239</td>
<td>54</td>
</tr>
<tr>
<td>2005/2006</td>
<td>16 Sept 2005</td>
<td>264</td>
<td>88</td>
</tr>
<tr>
<td>2006/2007</td>
<td>3-5 Oct 2006</td>
<td>213</td>
<td>75</td>
</tr>
<tr>
<td>2009/2010</td>
<td>6-7 Apr 2010</td>
<td>20</td>
<td>11</td>
</tr>
</tbody>
</table>

The mean density of *A. minutum* cysts per gm dry sediment presented in table 1 vary from results presented in Chapter IV. The densities presented in table 1 represent the mean of 13 sampling stations. During the winter periods of 2011, 2012 and 2013, the number of sampling sites was reduced and focused predominately on the western end of the channel. For this reason, mean *A. minutum* cyst densities in chapter IV were calculated from the latter set of stations to ensure comparability. The examination of *A. minutum* cyst densities found counts as high as 1680 cysts .g dry wt sediment⁻¹ during the dormancy period of 2003/2004. While in September of 2004, after the summer bloom, the highest count recorded for

Figure 3. *A. minutum* and *A. tamarense* cyst densities plotted against *Alexandrium* spp. cell densities (2003-2010).
Chapter VI

*A. minutum* was 301 cysts g dry wt sediment$^{-1}$. A similar pattern can be observed for *A. tamarense* cyst densities, although counts were relatively lower than *A. minutum*. As once again, during the same dormancy period, a maximum recorded count of 956 cysts g dry wt sediment$^{-1}$ was found to be substantially lower in September of 2004 (239 cysts g dry wt sediment$^{-1}$). The data derived from four surveys proceeding 2003/2004 all also signify low remaining densities. A time series record of *Alexandrium spp.* cell densities in the water column was subsequently derived and analysed. This data gave us an idea as to what was happening annually in the water column of the N. Channel in contrast to the sediment below. A bloom of 620,000 cells L$^{-1}$ was observed in the western half of the N. Channel in the summer of 2004. Yet during the same period in 2005, the maximum count recorded was only 31,000 cells L$^{-1}$. With the exception of the year 2009 cell densities were recorded annually, but never peaked above 55,000 cells L$^{-1}$.

**DISCUSSION**

Unfortunately harmful blooms leading to possible health hazards and great financial loss are commonplace at sites of cultivation. The monitoring of these natural phenomena is crucial in order to be able to predict bloom events and if possible, their intensity. *Alexandrium* blooms in the North Channel are an annual occurrence, yet the density of cells in the water column has substantially decreased since the year 2004. Cyst densities in the N. Channel have been found to be highly concentrated in spots and areas of maximum concentration can change from year to year. Portraying a similar scenario to the *Alexandrium* cell densities, a decrease in cyst stocks has also been observed since 2004, as there appears to have been very little recovery of *Alexandrium* seed beds after an exceptional bloom in 2005. Examining data derived since 2003 indicates that a decline in bloom intensity has coincided with a considerable reduction in the concentration of cysts in the seabed of the N. Channel. From this observation, it is tempting to suggest that the winter cyst densities in the sediment of the North Channel can act as an indicator for the size of the subsequent annual *Alexandrium* bloom in the water column.
REFERENCES


Overview

Toxic populations of *A. minutum* can impact severely on an ecosystem and pose a major risk to public health if not monitored and managed correctly. *A. minutum* is commonly found along regions of the European coastline and has a history of causing toxic blooms near aquaculture sites. A reliable method of prediction for these blooms could potentially lead to mitigation methods and help reduce the severity of their impact. In spite of numerous studies carried out on this species, we still know relatively little of its behavior and life cycle patterns. A greater understanding of its various life history stages and the factors which best control its initiation and termination could potentially lead to bloom prediction methods in the future. An increased level of monitoring in many areas has lead to an increased awareness of annual HAB events and cases of human related illness. The behavior of geographically isolated populations of *A. minutum* will vary with environmental conditions. This makes the development of a prediction method which can be applied across a wide geographical range extremely difficult. Yet, exploring the fundamental parameters which can influence the initiation, magnitude and termination of a bloom may help to reveal windows of opportunity which provide optimum conditions for bloom initiation and cell proliferation.

The main objective of this study was to investigate a toxic *A. minutum* population in an area which boasts an extensive aquaculture industry and to identify the main influences on its bloom dynamics. The south and southwest coast of Ireland has long been recognized as an area susceptible to destructive HAB events, but it is only within the last two decades that any significant studies have been carried out to investigate the reasons as to why harmful events are so common here. Since 2003, annual densities of *Alexandrium* cell and cyst populations within Cork Harbour have been recorded during summer and winter months respectively. Throughout this time, investigations into the toxic composition, genetic diversity and ecophysiology of *Alexandrium* spp. have been
carried out, in addition to an extensive study which successfully modeled the initiation of *Alexandrium* spp. blooms in Cork Harbour. The intention of this study was to contribute further to the existing database of *A. minutum* cell and cyst densities, whilst taking a closer look at their life history stages and at how both asexual and sexual reproductive phases can impact upon population dynamics. Bloom initiation was modeled based on the observed variations in environmental parameters of irradiance, temperature and tidal dilution, while sexual reproductive stages were used to simulate bloom termination. Aside from the role physiological changes play in *A. minutum* cell behavior and population dynamics, an attempt was made to determine the level of control parasitism exerted on the functional ecology of the population. As such observations in the natural environment require an accurate and consistent method of monitoring, this study also researched the comparability of the various methods commonly employed for the monitoring of HAB events, and determined the potential of more automated methods.

**Results summary**

**Monitoring methodologies for the quantification of *Alexandrium* spp.**
No significant difference was found between the use of imaging flow cytometry and Fluorescent In Situ hybridization (FISH) techniques when quantifying *Alexandrium* cells within heterogeneous samples. This is an encouraging result for the potential use of more automated methods in future monitoring programs. FISH was the most commonly employed technique for the enumeration of *A. minutum* cells during the two field surveys carried out in the North Channel area of Cork Harbour. Due to a diversity of *Alexandrium* spp. present in this area, this method allowed for the accurate discrimination of *A. minutum* cells within samples. The successful coupling of the FlowCAM with a specific oligonucleotide probe is a progressive result in the field of flow cytometry. Although initial efforts were not quantitatively accurate, it is a step towards the characterization and enumeration of individual species using automated methods. The FlowCAM also successfully illustrated a complete overview of the phytoplankton community structure, an essential feature when investigating responses in the water column to short-term
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environmental changes. Although automated methods hold great potential, the Utermöhl method proved to be the most accurate of all the techniques reviewed, highlighting the value of traditional microscopy in microalgae monitoring.

*A. minutum* excystment

The analysis of *A. minutum* cyst densities in the North Channel between 2003 and 2011 found a considerable reduction in the concentration of cysts in the seabed following the 2003/2004 winter period. As this decrease coincided with considerably lower vegetative (planktonic) cell densities during the same period, it was suggested that winter cyst density influenced the magnitude of the subsequent annual *Alexandrium* bloom in the water column. This idea was later shown to be incorrect when the highest *A. minutum* bloom recorded to date (>1.5 x 10^6 cells L^{-1}) in the North Channel was detected in 2011 and followed a low winter cyst density of 40 cysts g^{-1}. A relatively low bloom of *A. minutum* (30,000 cells L^{-1}) in 2012 then followed what was deemed to be a high cyst density of >1000 cysts g^{-1}. These results provide clear evidence that the intensity of spring/summer *Alexandrium* blooms in the North Channel is independent of the cyst density measured the previous winter. Highlighted also from these results is the need for extensive time series data sets when attempting to predict bloom dynamics. The six year data set presented in chapter VI was believed to represent a relationship between cyst density and bloom intensity, yet one additional year managed to contradict this theory. A cyst dynamics model also demonstrated that the cysts supplied by periodic intense blooms, found to occur with frequency of every 7-8 years in the North Channel, are not in themselves enough to allow the population to persist over long time scales (decades). With a degree of resuspension, the cyst input from interim blooms of lower density is however satisfactory to ensure the annual inoculation of the water column with *A. minutum* cells. A maturation time of the order of months suggests that the supply of cysts from one particular year seeds the bloom in the next, and possibly subsequent, years. Cysts held at a temperature of 15 °C produced excystment rates >50% within 160 days, while a decrease in 5 °C was found to increase maturation time by two months. In conclusion, these results suggest temperature plays a role in excystment and in turn, contributes to the timing of bloom initiation, while the cyst densities available for
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inoculation are not indicative of bloom magnitude.

*A. minutum* bloom dynamics

Intense blooms are defined in this study as cell concentrations which exceed 100,000 cells L⁻¹. Irrespective of their density, blooms of *A. minutum* in Cork Harbour are not observed in the water column for longer than, on average, 14 days. Cells may be present throughout the summer period in low concentrations, but the correct balance of environmental conditions appears necessary for cell proliferation. Based on optimum levels of photosynthetically active radiation and water temperature levels, a four month window of opportunity was determined for potential bloom development. Yet, a bloom dynamics model simulating the intense bloom years of 2011 and 2004 suggest maximum cell concentrations are highly influenced by rates of tidal dilution. In agreement with the observed data, the model accurately simulates the 14 day difference between the bloom peaks in 2004 and 2011. Both blooms peaked during the low spring tide in June, highlighting the influence of reduced tidal flushing on their development. This result concurs with a previous theory suggesting *A. minutum* blooms in Cork Harbour require at least ten days for development. A low spring tide around the time of the summer solstice creates an extended period of low tidal dilution which, as this study indicates, supports maximum cell growth.

An investigation into the ability of the parasitic flagellate genera *Amoebophrya* and *Parvilucifera* to regulate *A. minutum* bloom dynamics was unsuccessful due to the lack of observations of infected cells within the population. Both parasitic genera were detected in the water column of Cork Harbour using fluorescence microscopy and molecular analysis. While *Amoebophrya* was evidently infecting the dinoflagellate populations of *A. sanguinea*, *H. triquetra* and *S. trochoidea*, it remains unknown which species were host to *Parvilucifera*. *Amoebophrya* prevailed among up to 77% and 55% of the *H. triquetra* population in 2011 and 2012 respectively. Such high prevalence rates appeared to suppress bloom development, as cell densities dropped two orders of magnitude in 2011 after infection. In 2012, cell concentrations also fell substantially, yet managed to recover and reach the maximum recorded density within seven days. These results suggest high
parasitic prevalence rates can cause the repress of dinoflagellate bloom magnitude but their impact may only be temporary. Cross experimentation in culture also illustrated a degree of species specificity in the *Amoebophrya* sp. infecting the dinoflagellate hosts. PCR analysis linked an increase in the concentration of *Parvilucifera* with periods of maximum *A. minutum* cell concentrations in both 2011 and 2012, yet light microscopy failed to identify infections with the species. Analysis of a partial 18s rRNA gene fragment revealed the *Parvilucifera* sp. detected in Cork Harbour shared 100% identity with a strain of *P. sinerae* isolated from Arenys de Mar Harbor in Mediterranean waters (Figueroa et al., 2008). Although a relationship between *A. minutum* and marine parasites was not achieved, these results signify the ability of dinoflagellate populations, albeit at low concentrations, to succeed during infection.

**A. minutum** encystment

*A. minutum* planozygote cells identified by morphology were first observed in the water column two weeks prior to the bloom peak, indicating sexual reproduction within the population. They remained in the water column during the following three weeks, and on average represented 14% of the planktonic population. *A. minutum* bloom termination was simulated for both 2004 and 2011 using the bloom dynamics model. A gamete formation rate of 40% produced the best comparison between the simulated output and field observations. This level of sexuality was found capable of terminating an intense bloom of *A. minutum* within a three week period, with tidal dilution as the only other contributing factor. These results highlight the substantial role sexual reproduction plays in *A. minutum* bloom termination, and the ability of the population to reach its maximum cell density once sexuality has begun and when a proportion of cells are presumed to be no longer contributing to bloom development.

The 2011 bloom dynamics simulation suggests that with a gamete formation rate of 40%, the time required for a newly formed planozygote cell to progress to a hypnozygote state is five days, a life history stage which was reached by only 11% of the population. This result complies with a consistent observation of maximum cyst deposition (as measured by sediment traps in 2004, 2006, 2011 and 2012) occurring approximately one week after
the highest recorded *A. minutum* cell densities. The observed proportion of vegetative cells which underwent encystment during these years ranged from 0.7-5.1% and averaged at 2.5% of the population. In 2011, the average encystment rate measured at two locations in the North Channel was 2.4%. The bloom dynamics model, with a successful gamete collision rate of 20% (producing most accurate simulation of observed data) and a five day period before hypnozygote formation, found on average 3.6% of the *A. minutum* vegetative population encysted. Such comparable results suggest a degree of confidence in a gamete fusion rate of 20%.

*A. minutum* cyst distribution

Compared with two other regions of the Cork Harbour, the North Channel was found to contain the highest *A. minutum* cyst levels. The number of cysts recorded in an area to the south of the North Channel was one third of the density found in the North Channel, while densities were found to be over an order of magnitude lower in the main western region of Cork Harbour. Cyst ‘hotspots’ in the North Channel were predominately located in its western half due to the retentive nature of the area, but also in areas susceptible to eddy formations near the mouth of the channel. These results illustrate the influence of hydrodynamics on seed dispersal. Vertical cyst profiles taken during the winter periods of 2004 and 2011 show a large percentage of cysts residing within the top 1 cm of sediment, a result which can be related to post bloom situations. *A. minutum* bloom intensity was substantially lower during the years 2003 and 2006, and as a consequence cyst distribution profiles taken during both subsequent winter periods show a relatively homogenous pattern from 0 to 5 cm. Although cyst densities have shown to lack influence over bloom magnitude, bloom magnitude clearly affects the subsequent concentration of cysts within the upper layer of sediment.

Future developments and implications

The acquisition of automated methods in HAB monitoring programs may allow for more comprehensive observations of bloom dynamics during sampling periods. Increasing the spatial resolution of the sampling strategy would better our understanding of the behavior of the species in the area. Yet increasing spatial and temporal resolutions would involve the
analysis of an increased number of discrete samples, intensifying labor costs and extending the time lapse between sample collection and analysis. Imaging flow cytometry, allows for the rapid, cost-effective and practical routine monitoring of potential harmful algal species. Each sample run provides an overview of the entire phytoplankton community, representing the level of primary productivity within the region. Rutten et al., 2005 found phytoplankton population estimations calculated using imaging flow cytometry is capable of acting as reliable ecological indicators for overall phytoplankton status. Imaging flow cytometry has also been used in the past to indentify planozygote cells (Campbell et al., 2010), showing its potential as a method of indentifying the sexual reproduction phases within a population. Being able to rapidly detect life-history stages in real-time data would be a very useful addition for predicting bloom dynamics, as the onset of sexual reproduction is often an indication of an impending bloom peak (Kremp and Heiskanen 1999, Garcés et al., 2004). One of the limitations of the FlowCAM highlighted in this study was it its inability to reliable distinguish specific species based on its imaging analysis software. Yet Alvarez et al., 2014 describes an updated image analysis algorithm capable of automatically and accurately classifying cells. It numerically describes each image and a support vector machine algorithm separates the particles into 34 groups. The groups are then merged taxonomically into seven functional classes and four shape classes. This new methodology may help eliminate the inaccuracy associated with the FlowCAM’s original imaging software.

Within a region such as Cork Harbour where morphologically similar members of the *Alexandrium* genus exist, automated methods would require the application of oligonucleotide probes and molecular assays to allow for species discrimination. Before considering the FlowCAM as a method for *A. minutum* bloom monitoring, I believe its ability to quantify specific species using FISH probes needs to be revised. Other instruments such as the environmental sample processor (ESP) (Scholin et al., 2009) can be deployed in an area of interest for the continual enumeration and discrimination of species in the microalgae community based on its genetic material. An automated sampling device such as this which provides real time data would be extremely useful if located within or near aquaculture sites on the southwest coast of Ireland. Although
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expensive, it is a non-disruptive method which increases the statistical significance of cell concentration estimates and potentially acts as an early warning system forecasting the onset of HAB events. The imaging Flowcytobot is another instrument developed within the last decade which can be deployed in the water column and left unattended for months at a time (Olsen and Sosik 2007). It is essentially a submersible flow cytometer which works on the same principle as the FlowCAM. It has the ability to communicate with an onshore laboratory, allowing constant analysis of the phytoplankton community. Based on the workings of the FlowCAM, it is unlikely this instrument would be able to distinguish between morphologically similar species, and so would be unsuitable in an environment such as the North Channel.

This study has revealed that the magnitude of an A. minutum bloom event in Cork Harbour is predominantly controlled by meteorological and hydrographic conditions. Levels of both irradiance and temperature play an important role in cell growth, yet tidal dilution appears to be the crucial factor which allows for bloom development. This revelation could potentially lead to the development of a prediction method for HAB events in the region based on tidal range patterns. A data set dating back nearly two decades indicates intense blooms of A. minutum in Cork Harbour occur with a frequency of every 7-8 years. Although this study has made significant progress in determining some of the main environmental parameters which support increased cell growth, the factors preventing cell concentrations from reaching intense bloom status during the interim years remain unknown. A greater understanding of the processes whether physiochemical, environmental or biological, which suppress cell growth, is essential for the development of a prediction method and would require further investigation in the Cork Harbour area. Further research on this topic would include an investigation into the effect of irradiance variability on cell growth. Throughout chapters III-V, we discuss the influence of both temperature and irradiance on A. minutum bloom timing. There is a period of ~4 months when temperatures exceed 15 °C and a period of ~6 months when irradiance exceeds 100 µE·m⁻²·s⁻² in the water column, yet irradiance levels are highly variable during this time period. It would be of use for future prediction methods to establish a correlation between this variability and A. minutum cell densities.
The identification of sexual reproductive stages within the population provides potential as an indicator of the commencement of bloom demise. Gamete formation has shown its exclusive potential to impede further bloom development once it reaches a threshold level in this study. As gamete cells do not differ extensively in their morphological appearance to the vegetative cells (Figueroa et al., 2007), an alternative method of identification is needed. The use of immunochemical labeling to identify lectin molecules on the surface of the gametes may be a reliable method of detection, as lectin binding differences have been previously observed during life history transitions within phytoplankton groups (Greenway, 1986, Kremp et al., 2004). Although many factors have been suggested to play a role in gamete formation, more thorough investigations of the main influencing parameters on *A. minutum* gamete formation in Cork Harbour may also contribute to a prediction method. In addition, the effect of small scale turbulence on successful gamete collision and fusion may contribute to the current knowledge of their life cycle strategies. Although Wyatt and Jenkinson (1997) suggest gamete collision to be a ‘rare’ event in the water column, we have provided evidence in this study portraying it as frequent event regardless of cell density. It would be of interest to investigate the strategies employed by the gamete cells, whether physiological or physical, to locate sexual mating types.

Although this study originally set out to establish a relationship between marine parasites and *A. minutum* in the North Channel of Cork Harbour, no relationship between the populations was established. Both *Parvilucifera* and *Amoebophrya* sp. were present within this area while the *A. minutum* population appeared unaffected by their presence, yet this cannot be confirmed. Coats et al., 1996 suggested the species of *Amoebophrya ceratii* to be a species complex and express a degree of host specificity. Although we can only suggest the species of *Amoebophrya* we observed in the North Channel was that of *A. ceratii*, it did demonstrate signs of host specificity. Perhaps this prevented *A. minutum* from succumbing to the parasite. The next step of this study would be to sequence the genomes of the individual *Amoebophrya* strains infecting the three dinoflagellate hosts of *H. triquetra*, *A. sanguinea* and *S. trochoidea* to identify if they do in fact belong to the *A. ceratii* species complex. Another important aspect of a future study would be to determine the dinoflagellate sp. playing host to *Parvilucifera infectans*. As the majority of
our samples used for analysis were preserved, it might be of more benefit to focus on live samples for the detection of this parasite; cell preservatives may hinder the morphological identification of infected cells.

Previous studies have found *Amoebophrya* spp. to impact substantially upon dinoflagellate populations (Chambouvet *et al.*, 2008, Coats and Bockstahler 1994), yet its influence on the population dynamics of the three dinoflagellate hosts in Cork Harbour did not appear to be detrimental. Coats *et al.*, 1996 previously described a parasitic infection of >20% to be in an epidemic phase. With regard to our results, no major impact was observed on the dinoflagellate populations until the prevalence rates exceeded 50%. The rate of prevalence within a population is most likely related to surrounding environmental and hydrodynamic conditions, as more enclosed systems are more likely to harbour infections among a population due to a closer proximity between cells in the water column. I believe the concept of introducing species of either *Parvilucifera* or *Amoebophrya* as a biological control for HAB events would require much further consideration. If infection rates of >50% are required to induce a detrimental impact on a population, achieving such high prevalence rates through the introduction of the parasite to the environment bears a high level of impracticality. The effect the parasite genera would have on the general ecosystem would also require extensive examination.
CONCLUSION

The primary objectives of this study were to investigate the main influencing parameter on *A. minutum* bloom dynamics in Cork Harbour, Ireland with the long term goal of contributing to a reliable HAB prediction method in the area. The most important results of this study stem from the life cycle observations which have indicated a number of important points. These include;

- the intensity of *A. minutum* blooms is independent of the cyst density measured the previous winter
- periodic large pulses of encystment are not essential to ensure the annual inoculation and perpetuation of *A. minutum* cells in the water column
- field observations demonstrate directly that sexual reproduction has the potential to contribute substantially to bloom termination
- gamete formation can reach 40% day$^{-1}$ at which it can impede bloom growth
- model simulations show that and a five day time period between planozygote and hypnozygote formation fits field observations.

Due to the influence life-history stages exert over bloom dynamics, all of these results contribute significantly to a greater understanding of *A. minutum* HAB events in Cork Harbour. They also suggest life history phases may play a more crucial role in *A. minutum* population dynamics than once previously thought. The remaining question is, however, what factors induce sexuality in *A. minutum*. To test the idea that parasitism has an influence on bloom termination, an attempt was made in monitoring their dynamics in tandem with *Alexandrium*. However, questions remain concerning their impact which demands further research. The amenability of *Alexandrium* dynamics to modeling in terms of temperature and irradiance controlled growth proved excellent in predicting the timing of bloom initiation. Modeling itself did not provide information on bloom intensity, and suggests that in the first instance the relationship between nutrient supply and *Alexandrium* bloom intensity should be investigated. This study has also highlighted the potential of automated methods of monitoring and their advantages, and
Concluding remarks

disadvantages, over more traditional methods of monitoring. This study was based on weekly sampling through the summer months. Use of an automated sampler in providing samples with a higher frequency might also yield further insights into bloom dynamics.
REFERENCES


APPENDIX I

*Alexandrium* biomass model, Cork Harbour

1. General

The model was designed to describe the change in biomass of *Alexandrium* spp. in the North Channel of Cork Harbour. It was run using the Microsoft package Excel spreadsheet software, using a time-step of 1 day.

The model is based on the change in biomass (dX/dt) that results from the balance between in situ growth (µ) and tidal dilution (D)

\[
\frac{dX}{dt} = \mu - D
\]

Growth is estimated from the relationship between growth rate and both irradiance and temperature. These were determined in a previous research project in the laboratory and have been published by Ni Rathaille *et al.* (2009) and can be summarised as follows.

2. Parameters and constants

2.1 Maximum growth rate

The maximum growth rate (µmax) was determined by repeat growth rate experiments in batch culture. The system was set up by placing an initial inoculum into replicate (ca. 30) tubes. At each time interval, the biomass (cell density) was determined in three tubes which were then discarded. The data was best fit using non-linear regression analysis based on the following equations:

2.2 Temperature (T) controlled growth:

If T<15 °C then \( \mu(T) = \mu_{\text{max}} \times (0.1 \times T - 0.5) \)

If T> 15 °C then \( \mu(T) = \mu_{\text{max}} \)
2.3 Irradiance (I) control of growth

If I < 100 µM·m⁻²·s⁻¹ then \( \mu(I) = \mu_{\text{max}} \cdot (I/100) \)

If I > 100 µM·m⁻²·s⁻¹ then \( \mu(I) = \mu_{\text{max}} \)

In this situation, I is Photosynthetically Active Radiation (PAR). It is the mean daily average based on a 14:10 light:dark cycle, as these were the conditions under which growth rate was measured in the laboratory (see Ni Rathaille et al., 2009)

2.4 In-situ growth rate

The in-situ temperature and light controlled growth rate was calculated as follows:

If temperature is > 15 °C, then the growth rate = \( \mu(I) \).

If temperature is < 15 °C, the growth rate is calculated by multiplying \( \mu(T) \) and \( \mu(I) \)

\[ \mu(T,I) = \mu(T) \cdot \mu(I) \]

So, available PAR becomes the main limiting factor when temperatures exceed 15 °C, as it is believed this temperature is optimum for \( A. \) minutum growth. When temperatures do not exceed 15 °C, multiplicative limitation is used as both parameters influence cell growth and both need to be taken into consideration when calculating the net growth.

Note that the effect of nutrients is not included as the model was developed for predicting the initiation of Alexandrium blooms.
2.5 Tidal Dilution

2.5.1 Model domain

The model domain was the western half of the North Channel of Cork Harbour. This was chosen as field observations demonstrated that this is where *Alexandrium* blooms in Cork Harbour initiate.

Western Boundary: The western end of the Channel does not mix with the water in the main channel of Cork harbour which runs up to Cork from Cobh. A notable front is located here at high tide (this boundary dries out at low tide) and field measurements have shown little or no mixing across the front. Temperature, salinity plots of water sampled across the entire Cork Harbour show how distinctly different the water in the North Channel is from that of the western branch (Cosgrove *et al.*, 2014).

Eastern Boundary: At mean low tide, the water that is found in the North Channel is almost exactly equivalent to the volume of water in the model domain at high tide. On an ebb tide, the Ballinacorragh river flows directly out of the North Channel down East Passage. On a flood tide, the river flow is negligible and is pushed back towards Midleton. This river therefore does not affect the model domain. This is further witnessed by the salinity distribution in the North Channel (see Ni Rathaille, 2007).

2.5.2 Tidal dilution of the model domain

Tidal dilution was estimated using a DIVAST model of Cork Harbour run by Dr. M. Harnett, Engineering, NUI Galway. This physical 2D model was run by first filling the model domain with a passive tracer (e.g. dye) and run over time for varying tidal heights. Observed concentrations of dye were then plotted, and the initial slope of the decrease in concentration was used to estimate tidal dilution. This technique has certain limitations, but it does allow for the re-introduction of tracer on subsequent flood tides into the model domain.

The relationship between tidal range (H, expressed in m) and dilution (D, expressed d⁻¹) was non-linear, and can be described by the following equation:

\[ D = 0.0362 \times e^{(0.6842 \times H)} \]
The calculation for cell net growth \( \text{d}^{-1} \) is as follows:

\[
\text{Net growth} = \mu(T) \times \mu(I) \text{ OR } \mu(I) \text{ (depending on temperature)} - D
\]

Table 1 Physical and biological parameters held constant in each model run

<table>
<thead>
<tr>
<th>Physical parameters</th>
<th>Value</th>
<th>Units</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean water column height ((h))</td>
<td>2.0</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>Vertical attenuation of PAR</td>
<td>1 - 1.1</td>
<td>m(^{-1})</td>
<td>Taken as the mean of estimates derived from Secchi depth measurements made through one or multiple years</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biological parameters</th>
<th>Value</th>
<th>Units</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum growth rate ((\mu_{\text{max}}))</td>
<td>0.47-0.54</td>
<td>d(^{-1})</td>
<td>Key value in the model from sensitivity analysis (see Ni Rathaille 2007)</td>
</tr>
</tbody>
</table>

3. Variables

Table 2. Variable environmental parameters used in each sample run

<table>
<thead>
<tr>
<th>Variable</th>
<th>Units</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>°C</td>
<td>Temperature sensor moored in the North Channel</td>
</tr>
</tbody>
</table>
| Irradiance     | \( \mu \text{ M m}^{-2} \text{ s}^{-1} \) | a) Irradiance sensor (Licor) mounted at 3 m height on the shores of North Channel  
                 |                                             | b) Global radiation data from Valentia (Met Eirann)  
The derivation of a mean daily PAR in a 2 m water column, based on a 14 h light day, from hourly global radiation measurements in J cm\(^{-2}\) is given in detail in Ni Rathaille 2007 (p.183). |
| Tidal Range    | m              | Difference between high and low tide heights, obtained from tide tables based on heights at Cobh |
4. Cell Inoculum

The inoculum of cells was derived in a number of ways. In the initial runs of the model, the cell density was held at a very small density of 50 cells L\(^{-1}\) until net growth was positive. Subsequently, the water column cell density was derived from *Alexandrium* cyst density and the relationship between season and excystment of the hypnozygote cysts as described in Ni Rathaille and Raine (2011).

Using excystment and cyst density, it was found that reworking (resuspension) of sediment was a prerequisite for the successful prediction of a bloom. In a water column averaging at 2 m, this is a reasonable assumption. The precise details are described in Ni Rathaille (2007) but the general concept of the procedure is described below:

The original field measurement was the cyst density in the top 1 cm sediment. The sediment, however, only had a 2mm top oxic layer (a prerequisite for excystment) so only 1/5 of the cysts were used to provide the inoculum of cells in the water column. At the end of each month, the top 1 cm of sediment was mixed, and a new cyst density on the top 2 mm sediment was estimated. Excystment rate, and its use to estimate ‘new’ vegetative planktonic cells, has been described in detail by Ni Rathaille (2007).

5. Model Output

The model was run in Excel and a typical section of output is shown below. A series of anchored cells contained values held constant for each model run (see Table 1 above).
Figure 1. Section output of the 2011 *Alexandrium* biomass model. Constant and variable parameters are illustrated in the first three columns.
REFERENCES


