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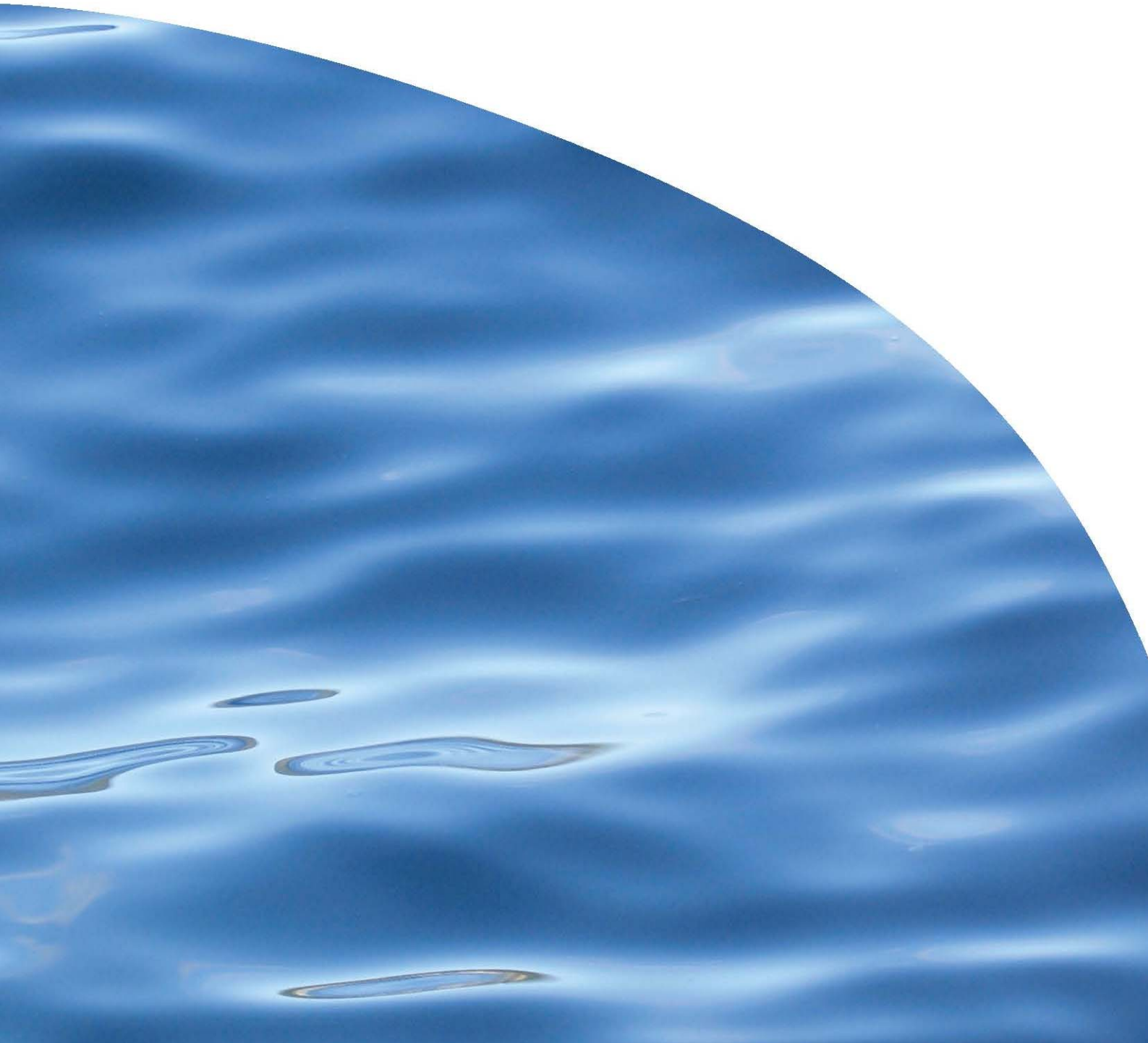
ALEXANDRIUM CATENELLA BLOOMS AND ASSOCIATED SAXITOXIN CONTAMINATION OF SHELLFISH, MARCH TO JUNE 2012



ALEXANDRIUM CATENELLA BLOOMS AND ASSOCIATED SAXITOXIN CONTAMINATION OF SHELLFISH, MARCH TO JUNE 2012

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EXECUTIVE SUMMARY

- A bloom of *Alexandrium catenella* began to develop in Opua Bay, Tory Channel, in late January 2012 and, from growth projections, it appeared likely there would have been a repeat of the widespread shellfish closures in 2011.
- However, bloom development was supressed by high winds in late February and early March which lowered temperatures and disrupted water column stratification.
- From late March to early April *A. catenella* increased again but it is believed that competition from a co-occurring dinoflagellate, and the lateness of the season, prevented it from becoming dominant.
- Low numbers of *A. catenella* and trace levels of saxitoxins (STXs) were observed at other locations in Queen Charlotte Sound in February, March and April and a single cell was seen in Port Underwood in April.
- A fluorescent dye (primuline) staining method, for counting *A. catenella* cysts in sediments samples, has been adopted which has increased the accuracy and speed of analysis. Estimates of cyst numbers have increased as a result.
- A cyst survey throughout Pelorus, Kenepuru and Queen Charlotte Sounds in February 2012 only identified *A. catenella* cysts in Queen Charlotte Sound and Tory Channel, with a distribution pattern similar to that seen in 2011.
- Very high numbers of cysts (15-35 $x10^6$ cysts / m^2 in the top 1cm) existed in the surface sediments of Opua Bay.
- Sectioning of a sediment core collected from Opua Bay, found high numbers of cysts to a depth of 6cm and significant numbers down to a depth of at least 10cm in the core. These results are preliminary and isotope dating of layers has yet to be carried out, however they suggest that the establishment of *A. catenella* in Opua Bay may not be very recent.
- Experimentally contaminated mussels from the Opua Bay buoy showed that the ratio between screen and confirmation toxicity analyses (0.35) was essentially identical to that measured the previous year (0.38). These data will be used in the validation documentation necessary to change regulatory procedures that will prevent unnecessarily long closure periods.
- *Alexandrium catenella* is entrenched in Opua Bay and annually recurrent blooms in the future will occur. We do not know for certain how long this has been going on and whether it will expand its range and spread to other regions within the Sounds, however this seems likely.

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1. INTRODUCTION

In late summer to autumn 2011, a bloom of the toxic dinoflagellate *Alexandrium catenella* developed in Queen Charlotte Sound (MacKenzie *et al.* 2011) accompanied by widespread contamination of shellfish with paralytic shellfish poisoning toxins (saxitoxins: STXs). The bloom was first detected in in early March 2011 and persisted though until mid-April 2011, although closures of commercial shellfish harvesting did not end in East Bay until 20 June 2011 due to the slow rate of toxin elimination. The maximum closure period was 97 days. Low numbers of *A. catenella* were first observed in Queen Charlotte Sound in May 2010 but the levels of STXs in shellfish were below the closure level of 0.8 mg/kg STX equivalents, and no further action was necessary at that time.

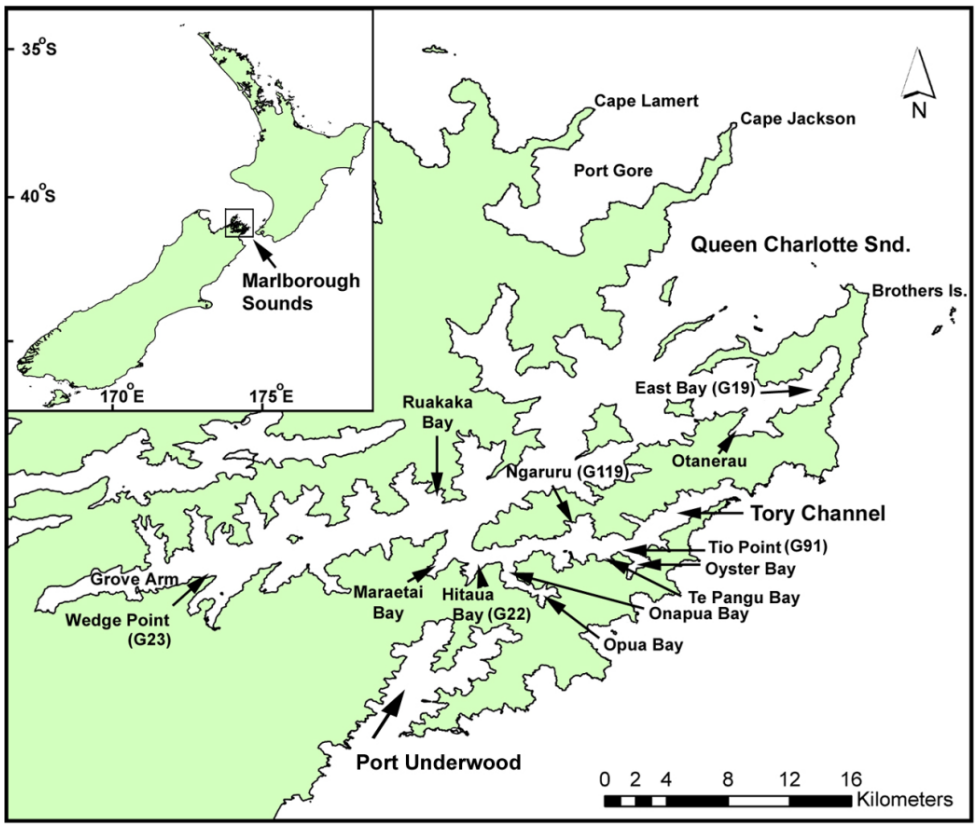

Figure 1 Locations in Queen Charlotte Sound referred to in the text.

A. catenella appeared to be a new component of the phytoplankton community in the Marlborough Sounds because this was the first occasion in 18 years of routine monitoring that this species had been observed, although it is common on the east coast of the North Island.

A. catenella is a prolific producer of resting cysts which over-winter on the sea floor and germinate to produce new blooms in subsequent years. After the 2011 bloom, surveys revealed that *A. catenella* resting cysts were widespread in the sediments around Queen Charlotte Sound and especially high numbers were found in Opua Bay off Tory Channel (Figure 1). Very high numbers of *A. catenella* (>4.0 x10^6 cells/litre) had been present in the water column of Opua Bay during the bloom and it appeared likely that this was the location where the bloom had originated and developed, before spreading to other areas.

A. catenella may be in the process of colonising the coastal waters of the northern South Island with implications for the shellfish aquaculture industry if, in the worst case, it becomes established and a chronic annual problem in the main mussel growing areas of Port Underwood, Pelorus Sound, Tasman and Golden Bays. If this occurs the industry may have to manage production around annual region-wide closures of 2-3 months in late summer and autumn.

With the support of the Marlborough Shellfish Quality Programme (MSQP) and reprioritisation of resources under Cawthron's Ministry of Science and Innovation (MSI)-funded Seafood Safety Programme, we have focused over the last year in closely monitoring the phytoplankton in Opua Bay to enable a better understanding of the ecology of *A. catenella* and to identify key environmental factors (such as temperature) that will assist in predicting its behaviour in the future. In addition, we carried out method development to improve our ability to rapidly count *A catenella* cysts in sediment samples. Using this method we carried out a comprehensive sediment sampling survey throughout Pelorus, Kenepuru and Queen Charlotte Sounds in February 2012, and sampled strata within a sediment core from Opua Bay to obtain an estimate of how long *A. catenella* has been resident in the region. We also carried out some mussel contamination trials to obtain data necessary to enable a revision of the closure and re-opening criteria for STX contamination, using the Lawrence chemical analysis procedure.

As expected, in mid-late January 2012 the cell numbers of *A. catenella* began to steadily increase in Opua Bay, and it looked as though a repeat of the extensive bloom and shellfish contamination of the previous year was likely. However, the weather conditions over the bloom period were different than the preceding year and a series of high wind events in late February and early March had a major impact on bloom development and this did not eventuate. A discussion of the various observations that were made and their implications for the future are presented here.

2. OPUA BAY MONITORING

In November 2011 a resource consent (mooring # 3317) was obtained to install a monitoring buoy for a period of five years (expiring 20 November 2016) in Tawa Bay, a small bay off Opua bay (Figure 2). The buoy was placed in this location to be out of the way of boat traffic and, although not ideally situated, it is in a position that is reasonably representative of the water column throughout the bay. Temperature sensors (HOBO Pendant Data loggers) at five depths, logging at 15 minute intervals, have been suspended from the buoy since December 2011.

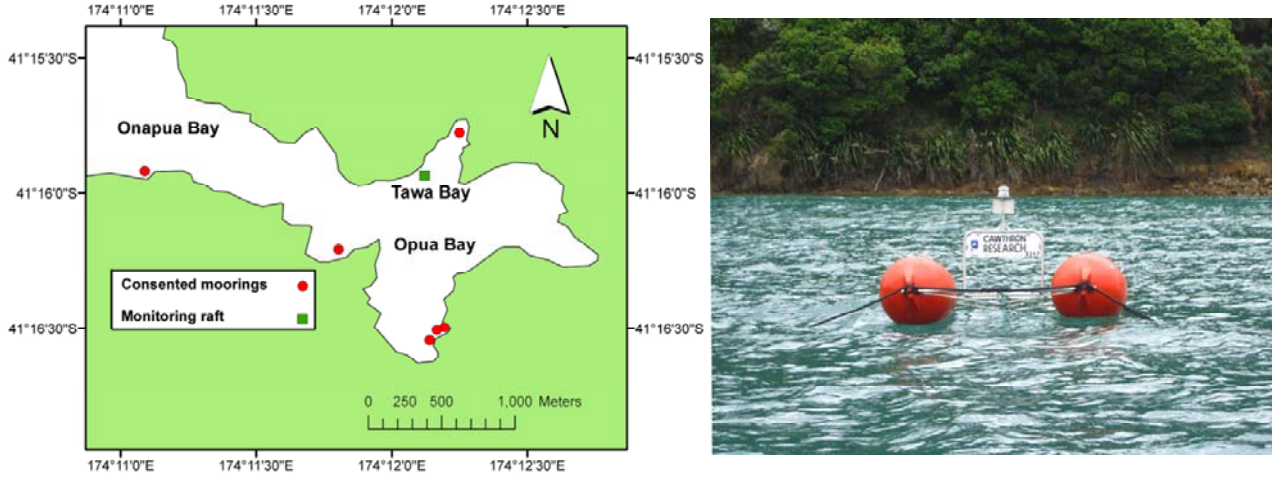

Figure 2 Location of the monitoring buoy in Opua Bay.

Lantern cages containing Greenshell™ mussels were suspended from the buoy during the early stages of the 2012 bloom, to obtain samples of contaminated material for experimental analysis.

Weekly collection of phytoplankton samples from Opua Bay has been carried out by MSQP since 21 September 2011. Sampling usually took place on a Wednesday, between the hours of 10.00 and 12.00, and samples were collected from 3m depth intervals (0m, 3m, 6m, 9m, 12m, 15m) using a van-Dorn sampling bottle. Microscope counts of *A. catenella* cells and documentation of other species co-occurring with it in these samples has been carried out. During the bloom period, from January to May 2012, reports were e-mailed to MSQP and affected shellfish farmers within 24 hours of the samples being collected. These reports contained a running tally of cell counts, graphical illustrations of trends (Figures 4 and 5), comments on the current situation and a tentative forecast of bloom progression. From 21 September 2011, when routine sampling in Opua Bay began, through to mid-January 2012, low numbers of *A. catenella* (100-200 cells/litre) were frequently observed in water samples from various depths (Figures 4A and 5A). From 18 January a steady rise in *A. catenella*

numbers occurred at 6-9 metres depth, accompanied with a rise in the numbers of another prominent dinoflagellate *Akashiwo sanguinea* (Figures 4B and 5B). The cell numbers of *A. catenella* reached a maximum on 7 March with highest numbers (229 x10^3 cells/litre) at the surface and numbers decreasing with increasing depth. High numbers of *A. sanguinea* (171 x10^3 cells/litre) accompanied *A. catenella* at the surface at this time (Figure 4B); though the numbers throughout the water column were lower (Figure 5B). Between 7 and 14 March there was a dramatic drop in the numbers of both species, before numbers began to increase again over the subsequent two weeks to a maximum on 4 April. Again, the numbers of *A. catenella* and *A. sanguinea* were highest at the surface at this time (108 x10^3 cells/litre and 156 x10^3 cells/litre respectively). Because of the larger cell size of *A. sanguinea* (Figure 3) this species substantially dominated the phytoplankton biomass.

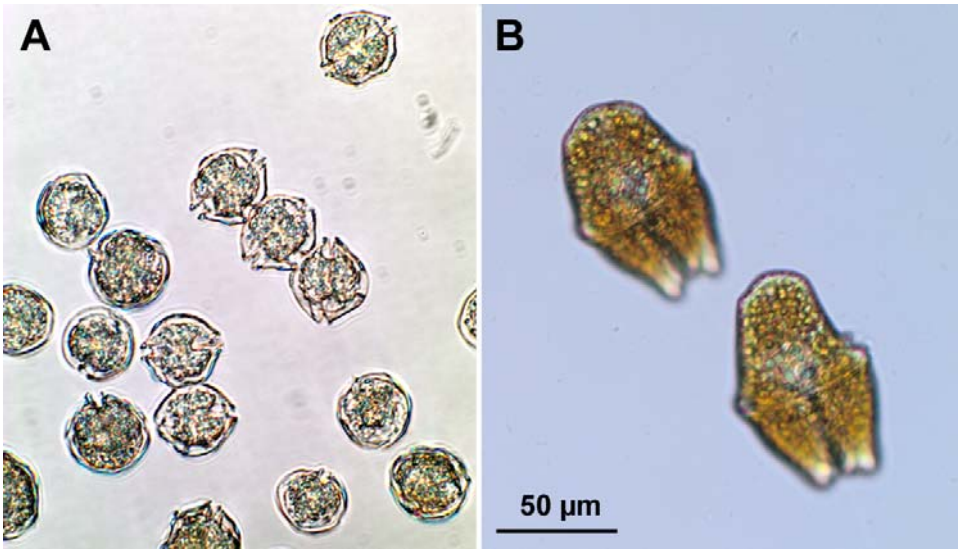

Figure 3 **A**. *Alexandrium catenella*. **B**. *Akashiwo sanguinea.* Both images are at the same scale.

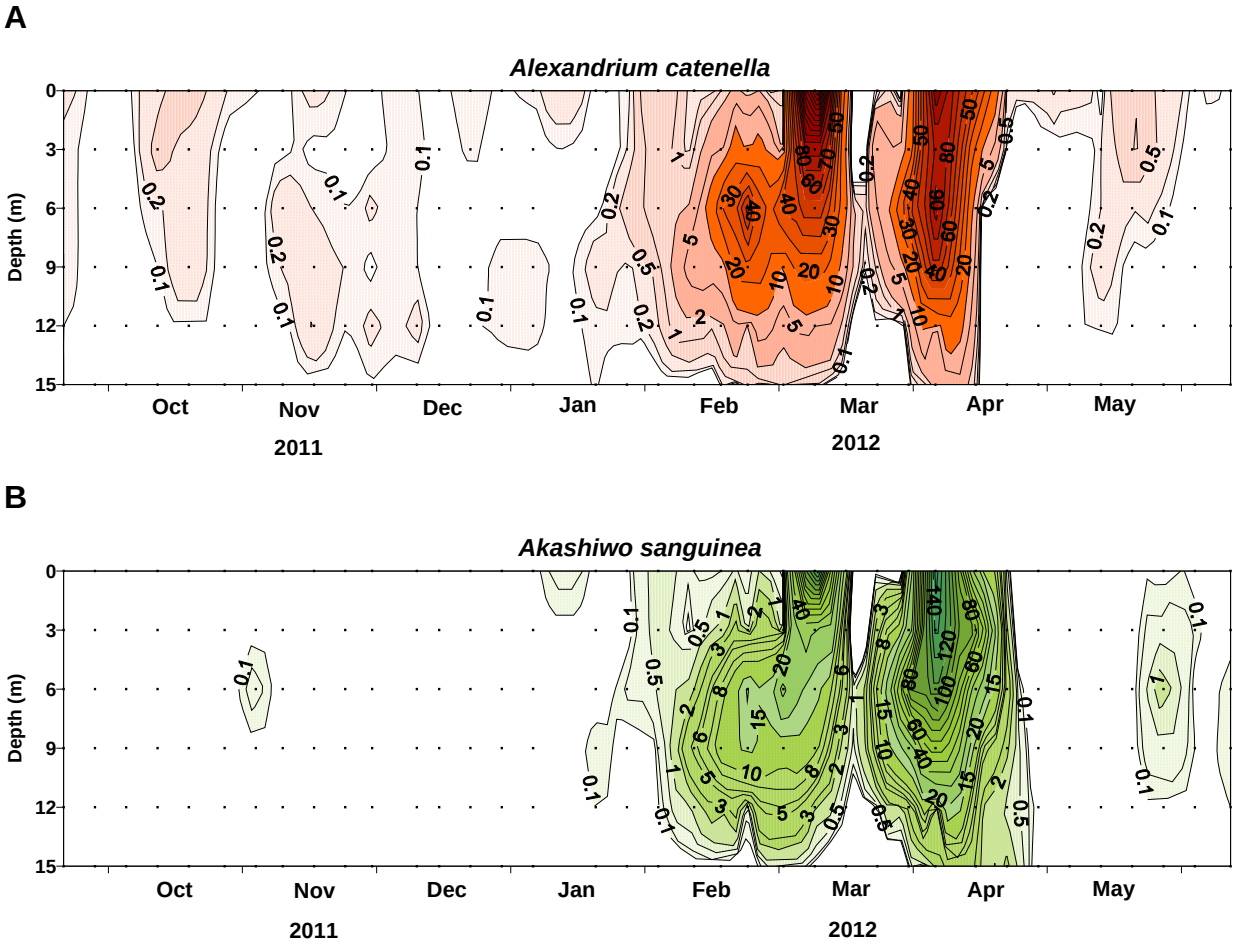

Figure 4 The distribution of dominant dinoflagellates (cells/litre x10^3) in the Opua Bay water column, sampled at 3 metre depth intervals, between 21 September 2011 and 7 June 2012. **A.** *Alexandrium catenella*. **B**. *Akashiwo sanguinea*.

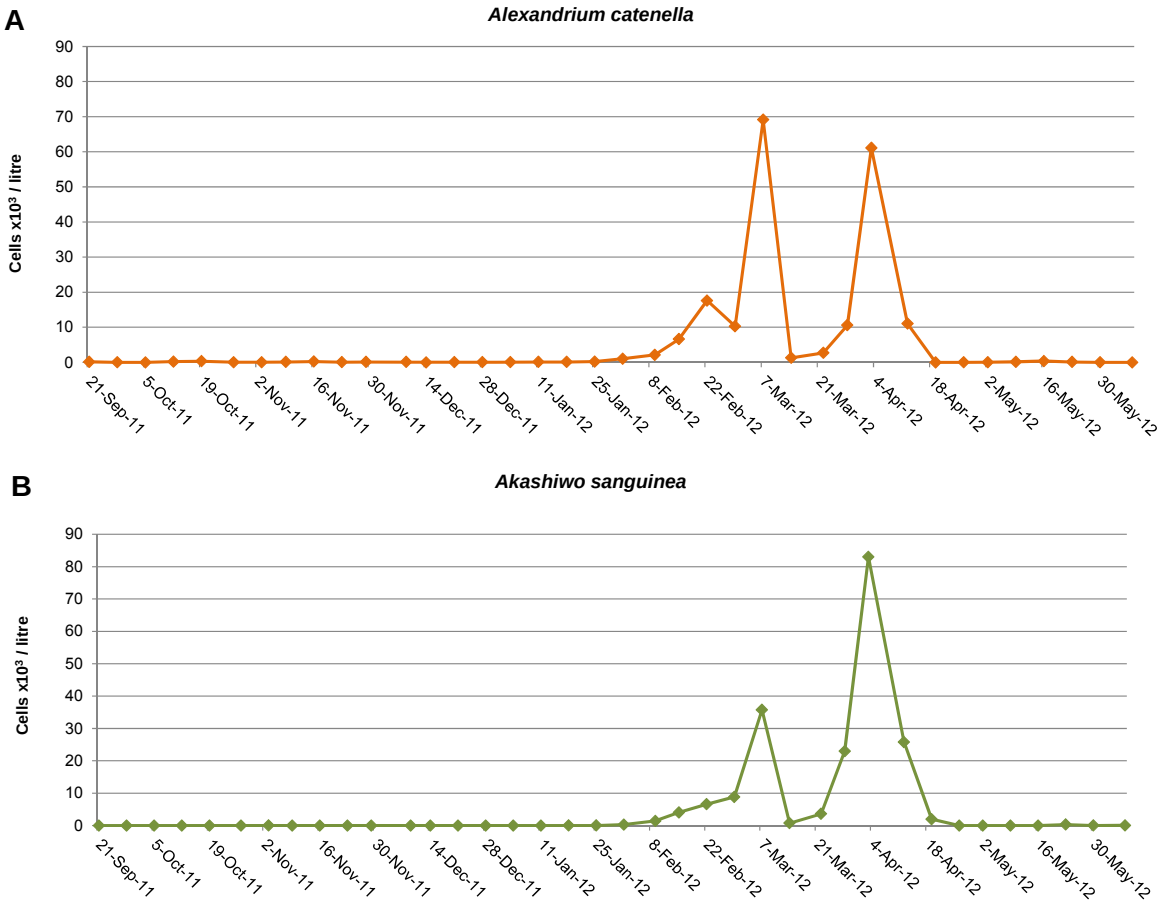

Figure 5 Depth averaged cell numbers (cells x10^3 / litre) of the dominant dinoflagellates in the Opua Bay water column between 21 September 2011 to 7 June 2012.

The initial increase in *A. catenella* cell numbers from late January to late February took place over a period when surface and bottom waters were gradually increasing in temperature and thermal stratification of the water column was strengthening (Figure 6B). On 23 February a low pressure system crossed the South island bringing with it high north-westerly winds to Cook Strait and the Sounds. This weather system coincided with a rapid decrease in the temperature (17.9 ºC on 23 February to 16.2 ºC on 29 February) of near-surface and mid-water column waters, followed by a precipitous drop in near-bottom water temperatures and thermal de-stratification of the water column on 4 March (Figures 6B and 6C). Thermal stratification was briefly re-established by 7 March, when the highest *A. catenella* numbers in near-surface waters were recorded (Figure 6D). On 3 March a deep low pressure region crossed the northern South Island (Figure 7) bringing with it a sustained period of strong south-easterly gales. Episodes of strong winds from the south east continued until 21 March and coincided with the period when the dinoflagellate community was slowly becoming re-established after its dramatic decline in mid-March. There were sporadic sighting of low numbers of *A. catenella* cells at other locations outside Opua Bay;

most notably at Tio Point (G91), Oyster Bay (Table 1) but the centre of the bloom, as in 2011, was in Opua Bay.

Table 1 Records of *A. catenella* in the Queen Charlotte Sound summer 2012 at sites other than Opua Bay.

Location	Date sampled	*A. catenella* (cells/litre)
Wedge Point	18 April 2012	100
Ruakaka Bay	4 January 2012	200
Ruakaka Bay	13 March 2012	500
Ruakaka Bay	3 April 2012	400
Te Pangu Bay	22 February 2012	200
Clay Point	10 April 2012	200
Tio Point	29 February 2012	200
Tio Point	7 March 2012	100
Tio Point	28 March 2012	600
Tio Point	3 April 2012	1500
Tio Point	9 May 2012	100
Horahora (Port Underwood)	18 April 2012	100

There was clearly a relationship between the thermal stratification of the water column and the dynamics of the *A. catenella* bloom in Opua Bay in late summer 2012, however, details of the hydrodynamics that lead to these changes is not known for certain. There are complexities due to the response of water circulation within Tory Channel itself under the influence of dramatically changing weather conditions which affect the water column structure in Opua Bay.

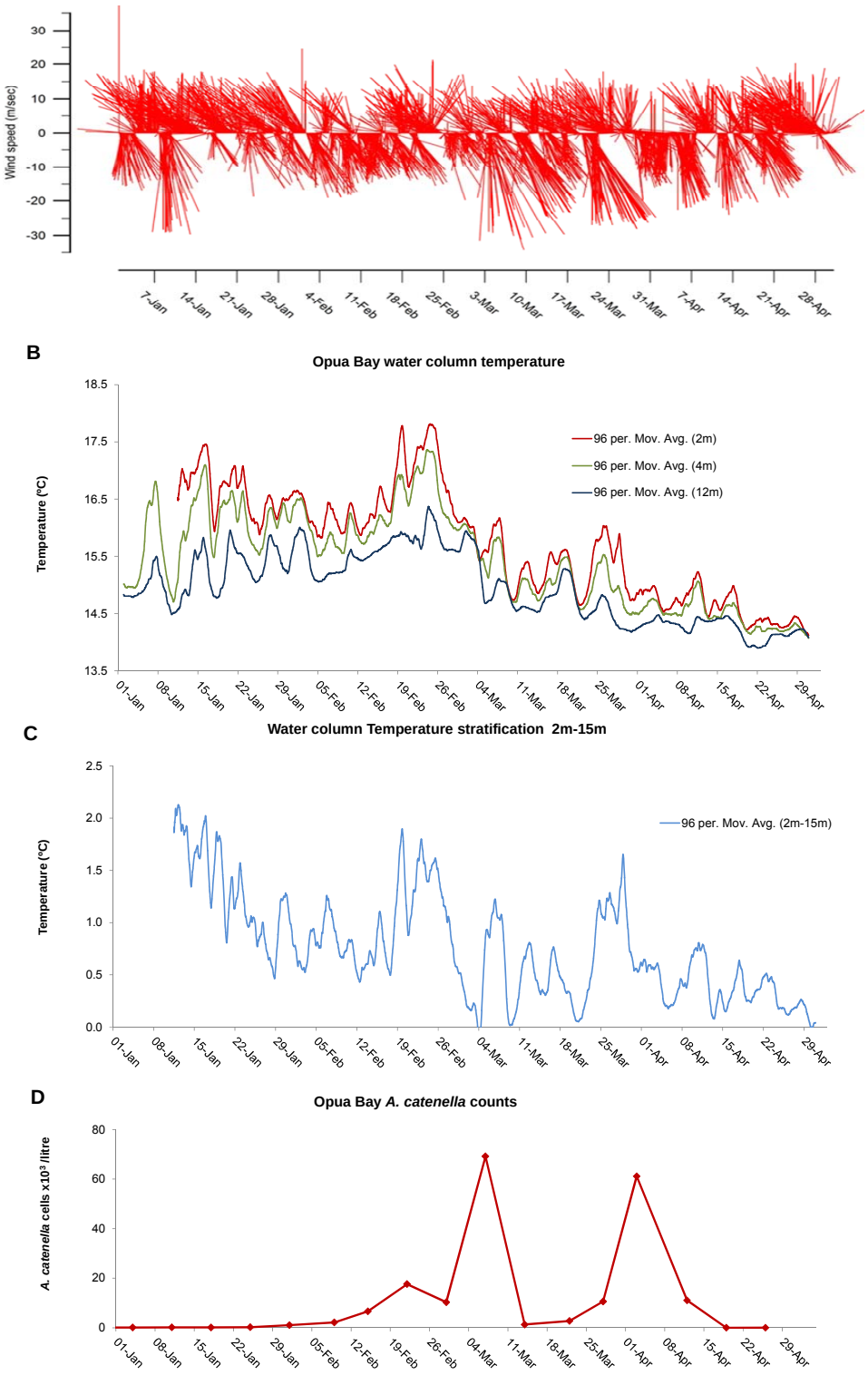

Figure 6 **A**. A vector plot of wind speed and direction records from the Brothers Islands, Cook Strait. **B.** Water temperatures at 2m, 4m and 12m recorded by *in situ* temperature loggers suspended from the Opua Bay monitoring buoy. **C**. Water column temperature stratification (2m -12m temperatures). **D**. *A. catenella* counts in the Opua Bay water column, 1January to 30 April 2012.

There was no evidence of the rapid appearance of *A. catenella* within the water column in the early stages of the bloom that might result from the sudden mass germination of cysts from the sediment. Judging from the occurrence of low numbers of cells in the water column, it is likely that cyst germination was occurring at a slow but steady rate for some months before conditions became optimal for the growth of motile cells in the water column. During January, under the influence of a warming, stratified water column *A. catenella* entered logarithmic growth phase (a population division rate of ~ 0.2 divisions/day) to achieve a maximum cell density of 229 $x10^3$ cells/litre at the surface on 7 March. At this time, due to a series of low pressure weather systems that crossed central New Zealand (Figures 7A and 7B), the water column rapidly cooled and there were episodes where thermal stratification was eliminated (Figure 6C). It is conceivable that under the strong north-westerly winds, from 23 to 25 February, the water column mixing was induced by down-welling of water in Opua Bay. Subsequent weather systems resulted in high gale-force winds from the south east which may have blown surface waters containing the dinoflagellates out of the bay and their replacement with deeper waters originating from Tory Channel. After 2 March thermal stratification and the dinoflagellate community within the bay became re-established, although by this time *A. sanguinea* had become dominant and the growth of *A. catenella* was suppressed. The reason for the sudden decline in dinoflagellate numbers after 12 April is unknown. Water temperatures were steadily declining and thermal stratification becoming weaker, several south-easterly wind events occurred and day length was decreasing at this time. These conditions may have supressed the growth of the dinoflagellate and possibly induced mass encystment and disappearance of the motile form from the water column.

From the cell count data from 18 January to 7 March (excluding the drop in cell numbers observed on 29 February, assuming this was a sampling issue) an *A. catenella* population division rate of 0.19 divisions/day or a population doubling time of 5.2 days was observed. This is similar to an *in situ* net growth rate of 0.24 divisions/day estimated for a population of *A. catenella* in Spain (Garcés *et al.* 2005). If the population had continued to grow at this rate, within another 20-25 days (*i.e.* by 27 March to 1 April) the population would have achieved a water column mean density of around 2.0 $x10^6$ cells/litre. During the 2011 bloom the highest cell numbers in Opua Bay were observed on 30 March with a water column mean cell density of 1.9 $x10^6$ cells/litre (cell numbers in surface waters were 4.4 $x10^6$ cells/litre). From this it can be assumed that if the stormy conditions in late February and early March 2012 had not occurred, the population would have continued to grow to achieve a density comparable to that of the previous year at the same time (*i.e.* at the end of March). Co-incidentally, high numbers of *A. catenella* were observed in samples from Port Fitzroy, Great Barrier Island, in late March 2012 (Appendix 1) and cultured Greenshell™ mussels from this location tested positive for saxitoxins.

It was clear from the progression of the 2012 bloom that weather conditions over the period played an important role in the eventual outcome. In the future, predictive models will need to draw on longer-term weather predictions to attempt forecasts. One of these may be the Antarctic oscillation index (Figure 7C) which is a measure of atmospheric pressure differences between polar and sub-polar regions that can be projected forward up to 14 days and relates to the passage of weather systems over central New Zealand.

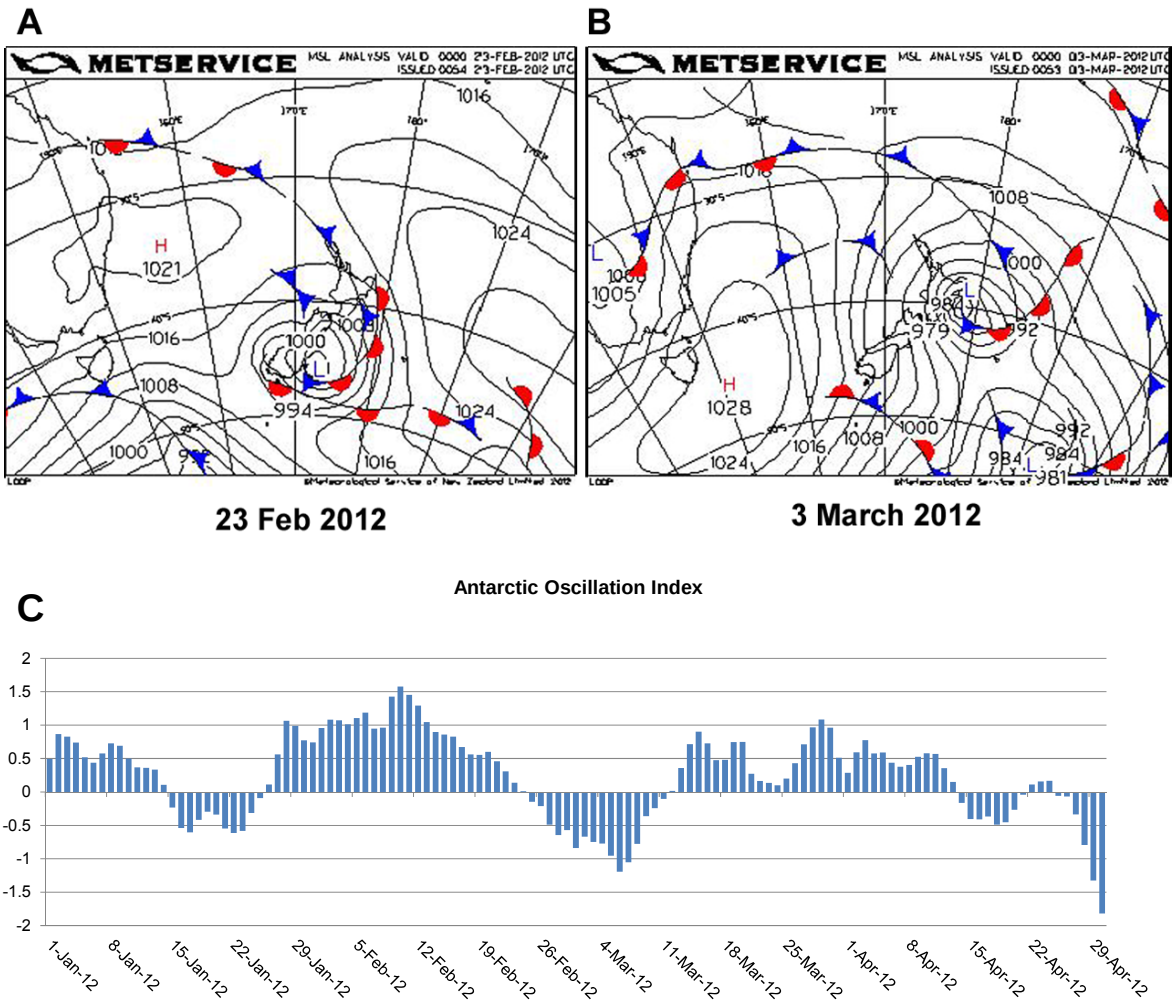

Figure 7 **A** and **B.** Two of the low pressure systems that crossed central New Zealand in February and March that brought strong winds to the Marlborough Sounds. **C.** The Antarctic oscillation index. The negative period during late February and early March 2012 was associated with the passage of these weather systems (Source: NOAA Climate Prediction Centre. ftp://ftp.cpc.ncep.noaa.gov/cwlinks/).

2.1. Why is *A. catenella* resident in Opua Bay?

It is not known how *A. catenella* first arrived in Opua Bay, but it is obviously a habitat that favours its growth and reproduction judging by the high numbers of resting cysts, which are the result of sexual conjugation, in the sediments. Opua Bay has many of the characteristics that are known to be conducive to the development of dinoflagellate-dominated communities. It is sheltered from all but the more extreme weather and it has weak currents and a long water residence time. It is sufficiently deep that a well thermally-stratified water column can develop in summer and, importantly, it is in close proximity to a source of naturally highly nutrient-enriched seawater from Tory Channel.

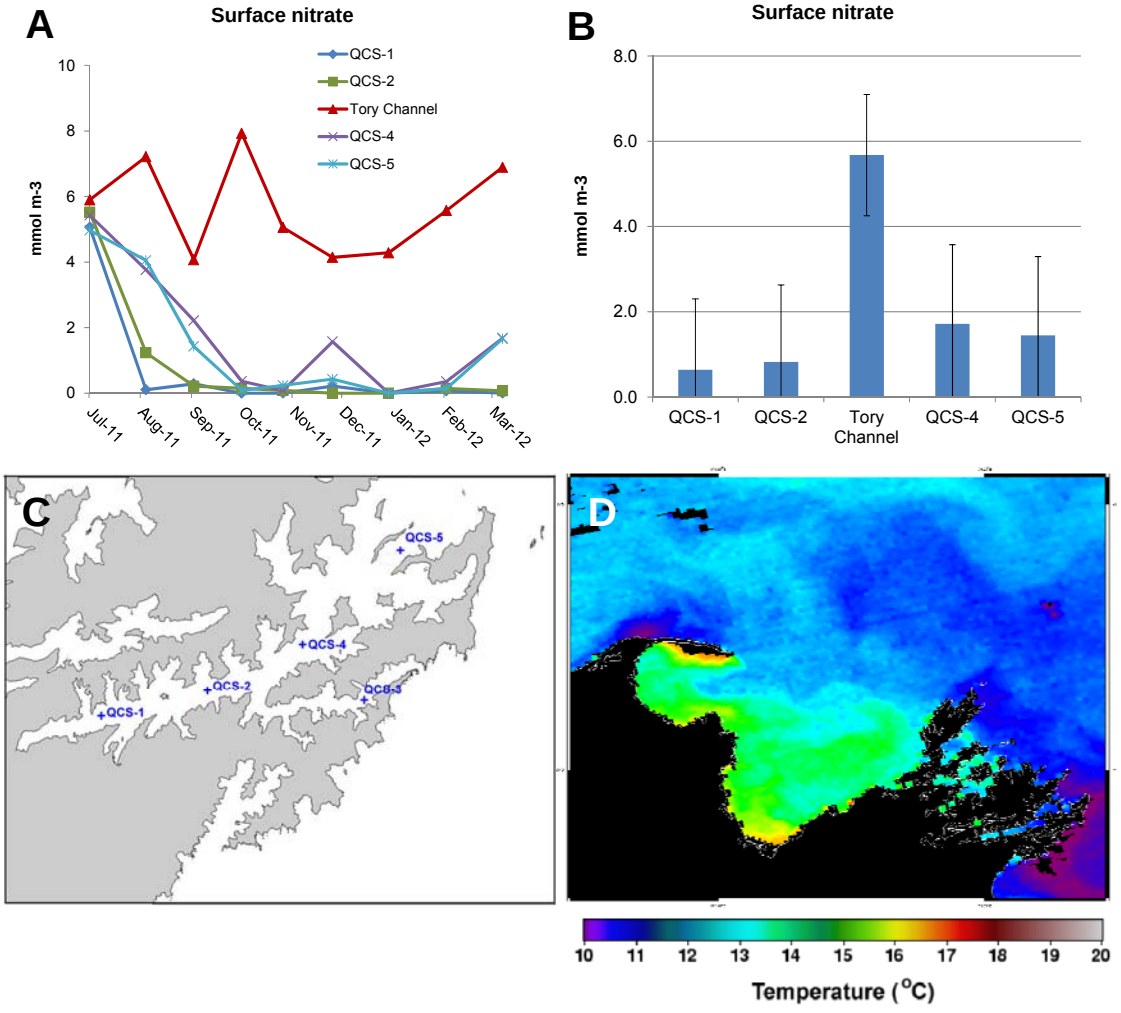

Figure 8 **A** and **B**. Nitrate concentrations in surface seawaters at various sites around Queen Charlotte Sound. **C.** Map showing the location of the sampling sites, QCS-3 is in mid-Tory Channel. **D.** Satellite sea surface temperatures image of the northern South Island taken on 18 February 2012.

Recent monitoring of seawater quality at five sites within Queen Charlotte Sound (Figure 8) has shown how important Tory Channel is to the fertility of the Sound. Because of the high currents in Tory Channel the water column is always well mixed and concentrations of nitrate are high throughout the year, even during summer when nitrate is all but completely depleted in near surface waters throughout the rest of the region (Figures 8A and B). The reason for these high nutrient levels in Tory Channel is due to persistent upwelling that takes place along the east coast of the Sounds in the vicinity of Tory Channel entrance. This cold (~10ºC) upwelled water is visible in satellite images of sea surface temperatures (Figure 8D). The upwelling is probably driven by the strong tidal currents in this area and prevailing north westerly winds that result in the surface waters being moved offshore and their replacement by deep nutrient-enriched water. This water is drawn into Tory Channel on each tide and there is a net residual flow which transports it through the channel into Queen Charlotte Sound. The conditions under which this nutrient-rich water gets transported into the Onapua/Opua side arm are not known for certain. The north west/south east orientation of the Inlet (Figure 1) suggests that down-welling/up-welling processes, driven alternatively by north west and south east winds, probably play an important role. A research priority during the next year will be to learn more about the hydrodynamics of the Onapua/Opua side arm and how this interacts with flows within Tory Channel. We hope to do this through modelling studies, the installation of current meters and monitoring of water chemistry and water column temperature profiles. The installation of a local wind speed and direction monitoring station would also be desirable.

3. SHELLFISH TOXICITY ASSOCIATED WITH THE 2012 *A. CATENELLA* BLOOM

The *A. catenella* bloom in 2011 was the first occasion when the new chemical testing programme based on the Lawrence pre-column oxidation HPLC method (Lawrence & Niedzwiadek, 2001, AOAC 2005) was used to manage a STX contamination event in New Zealand. This method was permitted for use by New Zealand Food Safety Authority (NZFSA) in late 2010 after a thorough evaluation of its performance (Holland *et al.* 2010). The method has two parts: a rapid screen test which enables highly sensitive detection of STXs but which, for technical reasons, inevitably produces over estimates of toxicity, and a more complex confirmation test which results in an accurate assessment of real levels of toxicity. The complexity and costs involved in the confirmation test means that this can only be used sparingly.

When the chemical testing regime was introduced, because there was no prior experience of its use in real toxic bloom events, a conservative level of ≥0.4 mg/kg STX equivalents was set by the Ministry for Primary Industry (MPI) as the closure criteria using the screen test. The level remained at the internationally accepted level

of ≥0.8 mg/kg STX equivalents on the results of a full confirmatory test. The reopening criteria was established as two consecutive screen tests <0.4 mg/kg STX equivalents, or confirmation tests <0.8 mg/kg STX equivalents. Data gathered during the 2011 event comparing screen and confirmation values showed the ratio of confirmation/screen values was constantly around 0.38. It became apparent that because low levels of toxicity hovered around the permissible screen test level (*i.e.* <0.4 mg/kg) for long periods during the toxin elimination phase, it was not possible to open areas without carrying out a full confirmatory test. In hindsight, because of the low confirmation/screen ratio, significant costs could have been avoided if re-opening was permitted on the basis of screen test results ≤ 0.8 mg/kg STX equivalents. To obtain more field data, to enable MPI to accept a screen test result of 0.8 mg/kg as the closure and opening criteria, Greenshell™ mussels were suspended in a lantern cage from the monitoring buoy in Opua Bay during the 2012 bloom. The mussels were obtained from the mussel farm in Hitaua Bay and analysis showed they contained no traces of STXs. Specimens (12) were sampled weekly and a composite extract analysed using the standard screen and confirmation tests.

After the placement of shellfish on the buoy on 15 February the mussels showed a level of STX equivalents of 3.8 mg/kg by the next week (22 February) and increased thereafter until reaching a maximum of 9.8 mg/kg on 7 March, coincident with the peak in *A. catenella* cell numbers (Figure 9). With the drop in *A. catenella* cells numbers after 7 March the shellfish toxicity also went into decline but lifted slightly in April as the bloom bounced back. There are several possible reasons why the toxicity did not increase proportionate to the increase in cell numbers during the second phase of the bloom. It may be that the shellfish, having become acclimatised during the initial bloom, were deliberately avoiding further toxin accumulation by ceasing feeding during the second phase, or, the high biomass of the non-toxic dinoflagellate *Akashiwo sanguinea* that occurred during this period (Figures. 4 and 5) diluted the effect of the increase in *A. catenella* cell numbers. A third, and probably important, reason was that by this time the lantern cages containing the mussels had become heavily fouled and water flow may have been significantly restricted. In any further experiments of this type this factor needs to be addressed if the aim is to obtain realistic estimates of the rates of toxin uptake and elimination.

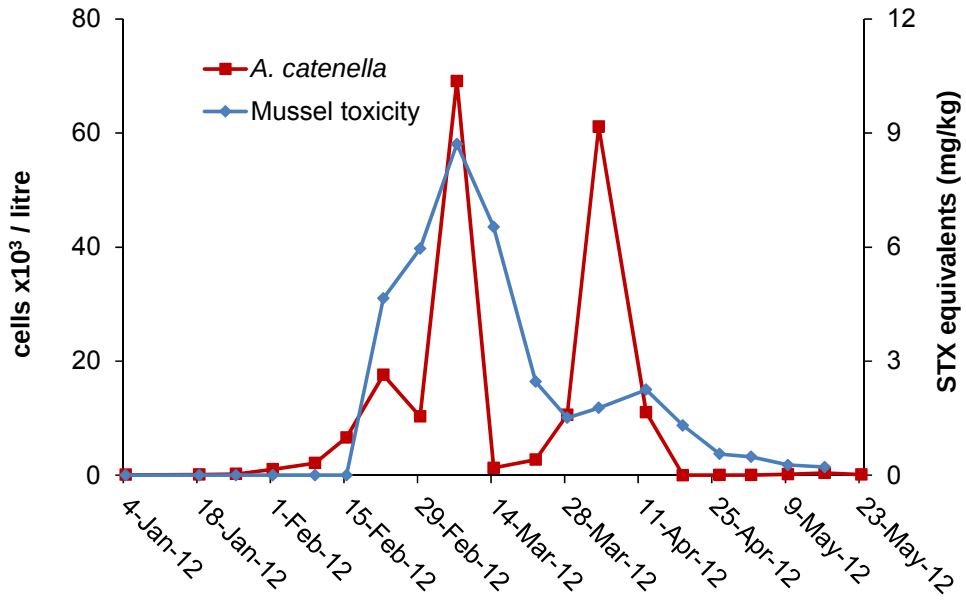

Figure 9 *A. catenella* cell numbers in the Opua Bay water column and PSP-toxicity screen levels (STX equivalents) in Greenshell™ mussels suspended from the monitoring buoy at 3 metres depth.

The toxin analysis of the mussel samples from Opua Bay in 2012, showed that the mean ratio (Table 2) between screen and confirmation toxicity tests (0.35 ± 0.04) was very similar to the mean ratio observed in 2011 (0.38 ± 0.04). This was expected because the toxin profiles in the dinoflagellate and the contaminated shellfish (Figure 10) were essentially identical in both years. The toxin profiles were dominated by the low toxicity analogues C1,2 and GTX5 (B1), whereas most of the calculated toxicity was contributed by GTX 1,4 and low to trace levels of other higher toxicity analogues (GTX 2,3; STX; dcSTX *etc*). The highest toxicity level in the Opua Bay mussels (9.8 mg/kg STX equivalents) was around 25 times the regulatory level of 0.4 mg/kg STX equivalents, but in other areas of Queen Charlotte Sound (Tio Point and East Bay) only low-trace levels were observed (Table 3). Only one sample (Tio Point, 18 April 2012) exceeded the regulatory level on the screen test by a small margin.

Table 2 Results of PSP-toxin analyses on Greenshell™ mussels from the Opua Bay monitoring site.

Sample date	Screen	Confirmation	Confirmation/Screen
	STX equivalents (mg/kg)		
22 February 2012	3.87	1.28	0.33
29 February 2012	5.47	1.64	0.30
7 March 2012	9.82	3.04	0.31
14 March 2012	8.04	3.04	0.38
23 March 2012	2.48	0.80	0.32
28 March 2012	1.51	0.62	0.41
3 April 2012	1.45	0.49	0.34
12 April 2012	2.59	0.82	0.32
18 April 2012	1.23	0.42	0.34
26 April 2012	0.46	0.20	0.43
9 May 2012	0.17	0.06	0.37
Mean ± stdev			**0.35 ± 0.04**

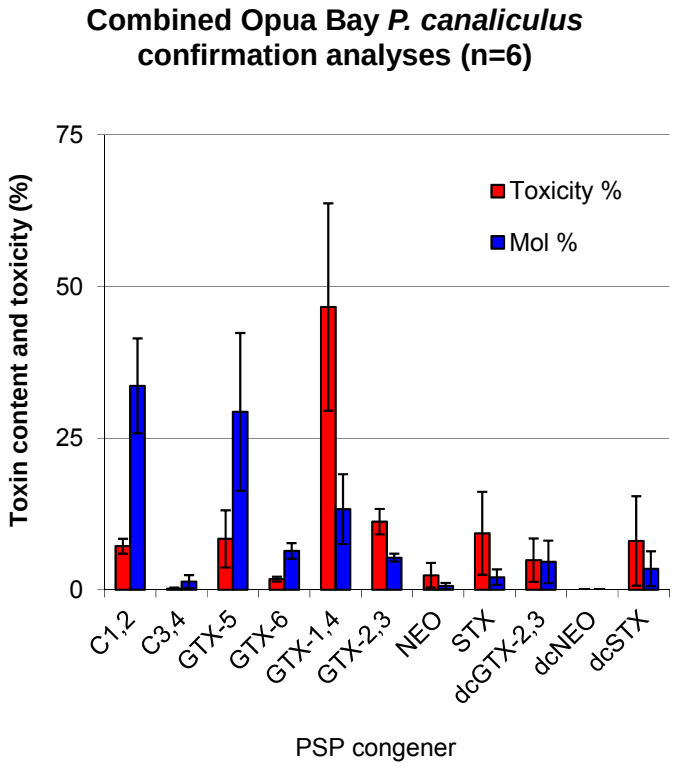

Figure 10 Contribution to the toxin content and toxicity by various STX analogues in Greenshell™ mussels from the Opua Bay buoy.

Table 3 Results of PSP-toxin screen analyses (Lawrence method) from Queen Charlotte Sound monitoring stations February to May 2012.

Location	Sampling date	Screen	Confirmation
Tio Point (G91)	1 February 2012	ND	-
Tio Point (G91)	9 February 2012	ND	-
Tio Point (G91)	15 February 2012	ND	-
Tio Point (G91)	22 February 2012	ND	-
Tio Point (G91)	29 February 2012	≤0.11	-
Tio Point (G91)	7 March 2012	≤0.19	-
Tio Point (G91)	14 March 2012	ND	-
Tio Point (G91)	22 March 2012	≤0.31	-
Tio Point (G91)	28 March 2012	≤0.17	-
Tio Point (G91)	3 April 2012	≤0.34	-
Tio Point (G91)	12 April 2012	≤0.26	-
Tio Point (G91)	18 April 2012	≤0.48	0.15
Tio Point (G91)	26 April 2012	≤0.24	-
Tio Point (G91)	2 May 2012	≤0.19	-
Tio Point (G91)	9 May 2012	≤0.10	-
East Bay (G19)	1 February 2012	ND	-
East Bay (G19)	9 February 2012	ND	-
East Bay (G19)	15 February 2012	ND	-
East Bay (G19)	22 February 2012	ND	-
East Bay (G19)	29 Febryary 2012	ND	-
East Bay (G19)	7 March 2012	ND	-
East Bay (G19)	14 March 2012	ND	-
East Bay (G19)	22 March 2012	ND	-
East Bay (G19)	28 March 2012	≤0.15	-
East Bay (G19)	3 April 2012	≤0.19	-
East Bay (G19)	12 April 2012	ND	-
East Bay (G19)	18 Apr 2012	ND	-

ND = not detected

4. *ALEXANDRIUM CATENELLA* CYST ANALYSES

4.1. Application of the primuline cyst-staining method.

Counting *A. catenella* cysts involves washing sediment samples through various size sieves to eliminate large and small particles, and microscopically examining the size fraction in which the cysts are contained. Unfortunately, there is a limit to what can be achieved by sieving, as there are many types of particles (*e.g.* sand grains, pollen, detritus) which fall within the size range of cysts. Under the microscope these particles form a crowded background against which it is difficult to quickly identify and count the cysts (Figure 11A). Yamaguchi *et al.* (1995) developed a fluorescent staining method that enables the semi-specific staining of *Alexandrium* species cysts which highlights them against the non-stained background (Figure 11B).

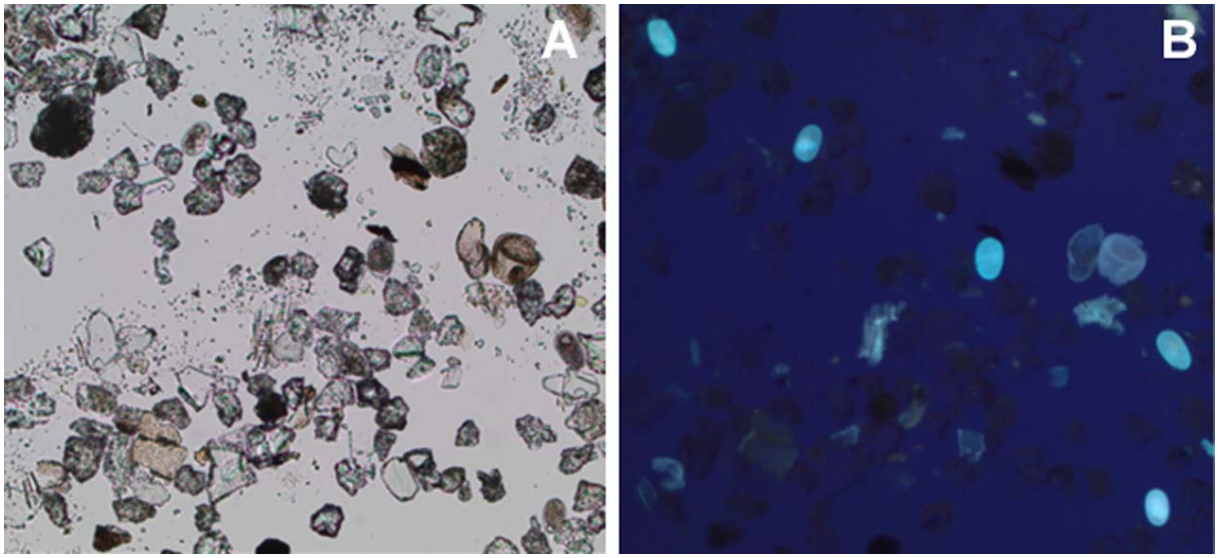

Figure 11 The same microscope field photographed under normal white light illumination (**A**) and under ultraviolet illumination (**B**). The blue oval objects are *A. catenella* cysts.

Over the 2011-2012 summer we successfully developed and tested a protocol (Appendix 2) for using the Yamaguchi method for the analysis of Marlborough Sounds sediments. This method considerably improved the speed and accuracy of cyst counts and the method was used in subsequent cyst surveys and analysis of sediment cores.

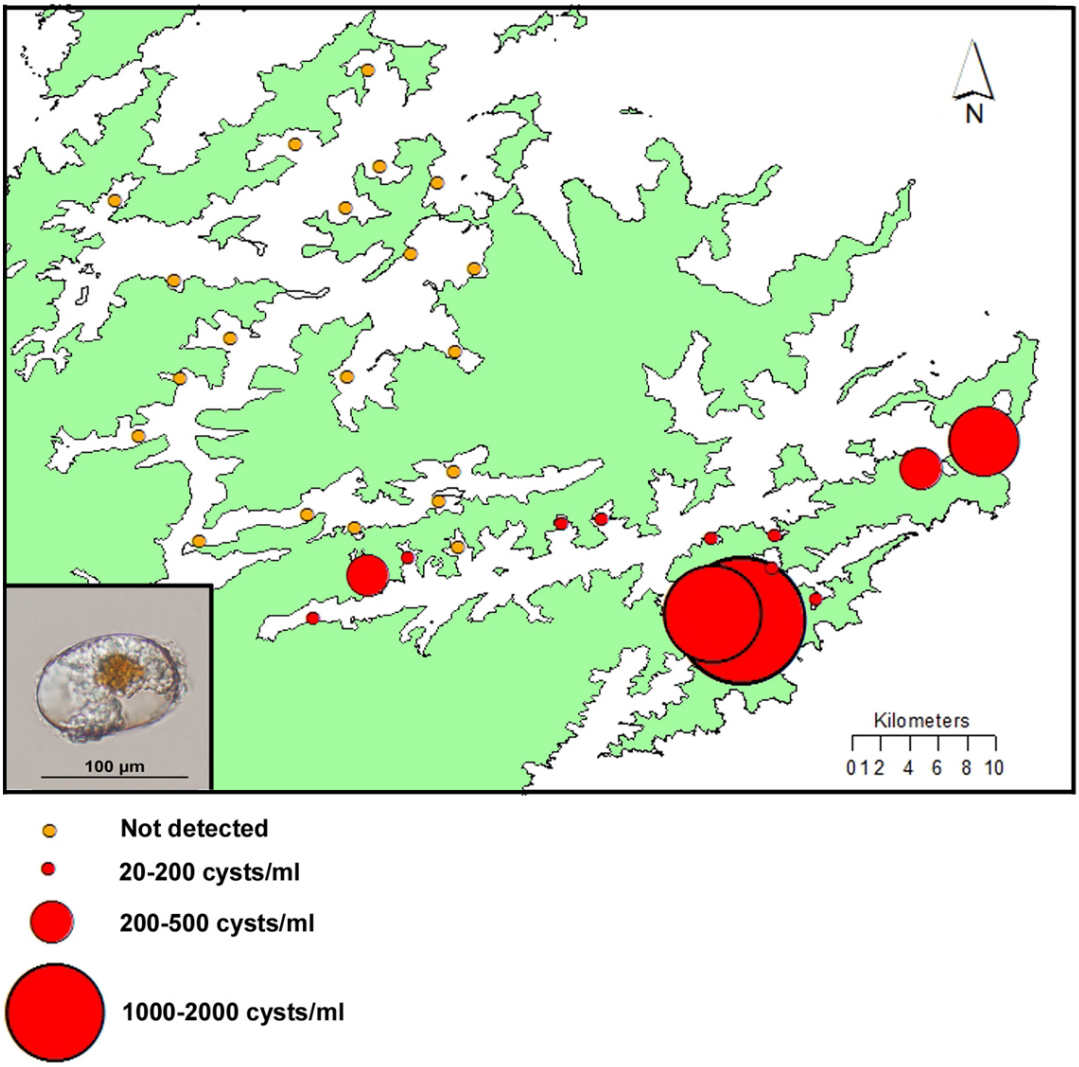

Figure 12 A survey of *A. catenella* cyst numbers in Pelorus and Queen Charlotte Sounds, 15 to 16 February 2012.

4.2. *Alexandrium catenella* cyst survey

In mid-February 2012 a survey of *Alexandrium catenella* cysts was carried out over two days (15 and16 February 2012) in Pelorus and Queen Charlotte Sounds. The top 1cm slices from 3 replicate cores, taken from sediment samples collected using a van Veen grab sampler, were combined. Samples were processed and stained with primuline fluorescent dye, according to our protocol (Appendix 3). The limit of detection using this method was 12.5 cysts/ml. Nineteen sites in Pelorus and Kenepuru Sounds and twelve sites in Queen Charlotte Sound were sampled (Appendix 3). No *A. catenella* cysts were observed in any samples from Pelorus and Kenepuru Sounds (Figure 12). The distribution pattern of cyst numbers in Queen Charlotte Sound was essentially the same as that observed in a similar survey carried out in June 2011 (MacKenzie *et al.* 2011), however, the estimates of numbers were

higher in the 2012 survey (Appendix 3) because of the use of the more sensitive primuline staining method. The highest cyst numbers were found in samples from Opua Bay at between 1,567-3,550 cyst /ml. These numbers represent around 15-35 $x10^6$ cysts / m^2 in the top 1cm of the sediment surface. These values are higher, but comparable, to the highest levels found in a few locations during a recent *A. catenella* cyst survey of Puget Sound, Washington, USA (Puget Sound Alexandrium Harmful Algal Bloom programme: (https://catalyst.uw.edu/workspace/banasn/14943/82760).

Numerous other cyst species were observed during these surveys, including several that could not be positively identified. The sediments within the numerous embayments off Queen Charlotte Sound support more abundant and diverse cyst communities than similar environments in Pelorus Sound. This probably reflects the different salinity regimes and sources and ratios of inorganic nutrients (N,P and Si) that provide more favourable dinoflagellate habitats in Queen Charlotte Sound. It is likely that an important factor in the propensity for Queen Charlotte Sound to experience a greater incidence of flagellate blooms than Pelorus Sound, relates to the oceanic origin of the seawater and the relatively small amounts of freshwater that flow into it.

4.3. *Alexandrium catenella* cysts in a sediment core from Opua Bay

During the February cyst survey a core was taken from a van Veen grab sample and frozen before extrusion and sectioning into 1cm slices from the surface to 10cm depth. These slices were sieved, primuline-stained and the numbers of *A. catenella* cysts counted. High numbers of cysts (>1000 cysts/ml) were found in this core sample to a depth of 6cm, and significant numbers in deeper layers down to a depth of at least 10cm (Figure 13). The significance of this observation is still debateable because it has not yet been possible to obtain estimates of the age of strata in the Opua Bay sediments by an independent radio-isotope (^{210}Pb) dating technique. However, it seems possible that *A. catenella* cyst deposition has been occurring here for some time.

The Opua Bay sediments are composed of soft, fine grained silt and appear to contain little in the way of macrofauna, but, given the high productivity of the bay and organic enrichment of the sediments, it is likely they contain abundant meiofauna communities (0.1mm to 1mm animals such as worms and crustaceans) that can rapidly turn over the surface layers of sediment and transport cysts to greater depths. Even if there is significant bioturbation of these sediments it seems unlikely that the presence of cysts, to at least 10cm depth, could be the result of their transport over a period of only a few years. Further sampling is planned to obtain deeper (~30cm) cores and have isotope dating of strata carried out alongside the cyst counts. This should enable

more accurate estimates of when the first *A. catenella* cysts first appear in the sedimentary record of Opua Bay.

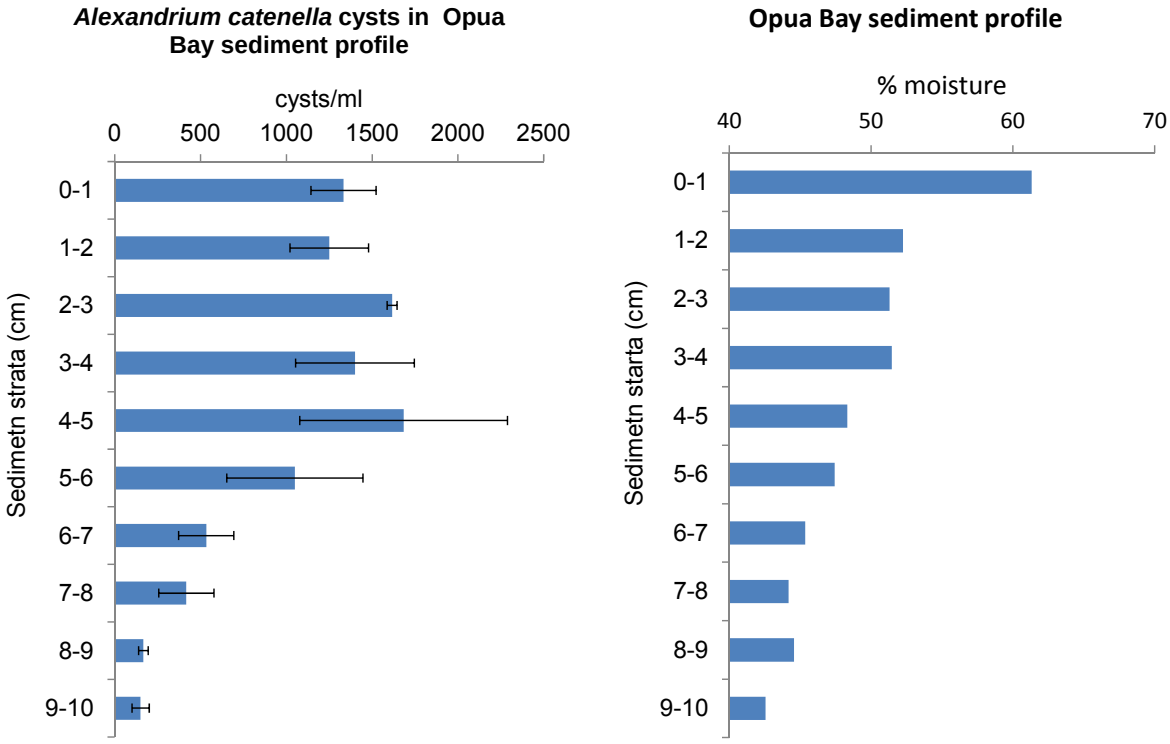

Figure 13 *A. catenella* cyst numbers and moisture content of 1cm layers within a core sample collected from Opua Bay.

A similar study of recent dinoflagellate cyst distributions was carried out by Irwin *et al.* (2003), who attempted to date sediment strata in cores from several locations around New Zealand and relate these to the distribution of cysts of the toxic dinoflagellate *Gymnodinium catenatum*. In that case ^{210}Pb dating showed that the depth of the surface mixed layer in sediments in Wellington Harbour was around 6cm. In the Hokianga and Manukau Harbours 10cm depth in the core represented a date within the early- to mid-1980s. If these dates are any guide *A. catenella* may have been blooming and depositing cysts in Opua Bay for at least several decades.

4.4. Cyst germination experiments

Several experiments were carried out to assess the viability of *A. catenella* cysts in the sediments from Opua Bay and their potential to initiate blooms. The density centrifugation technique of Bolch (1997) was used to harvest quantities of live cysts from sediment samples for the experiments. This method made the picking out of individual cells much quicker and easier than it would otherwise have been, but

attempts to use it for quantifying cyst numbers were less successful, as the proportion of cysts recovered from a sample was unpredictable. In the experiment, 30 cysts were individually picked out and placed in culture wells, incubated at 18ºC and examined daily for germination. Within 6 days over 90% of cysts had hatched, and after a further 30 days all had hatched. Of the cysts that germinated 83% successfully produced cultures of motile cells. These experiments showed that the majority of resting cysts in the top 1cm of the Opua Bay sediments (~15-35 x10^6 cyst/m^2) are capable of germinating and seeding new blooms under the right conditions.

4.5. Experiments to evaluate the use of quantitative PCR for the enumeration of *A. catenella* cell numbers in natural samples

We carried out a series of experiments to apply the use of quantitative polymerase chain reaction (qPCR) technology for the identification and enumeration of *A. catenella* cells in water and sediment samples. This technology may be applied in the future for use in automated *in situ* sampling devices and for rapid environmental surveys (*e.g*. Erdner *et al*. 2010). We achieved good progress in setting up the assay protocols and defining some important parameters essential for their application.

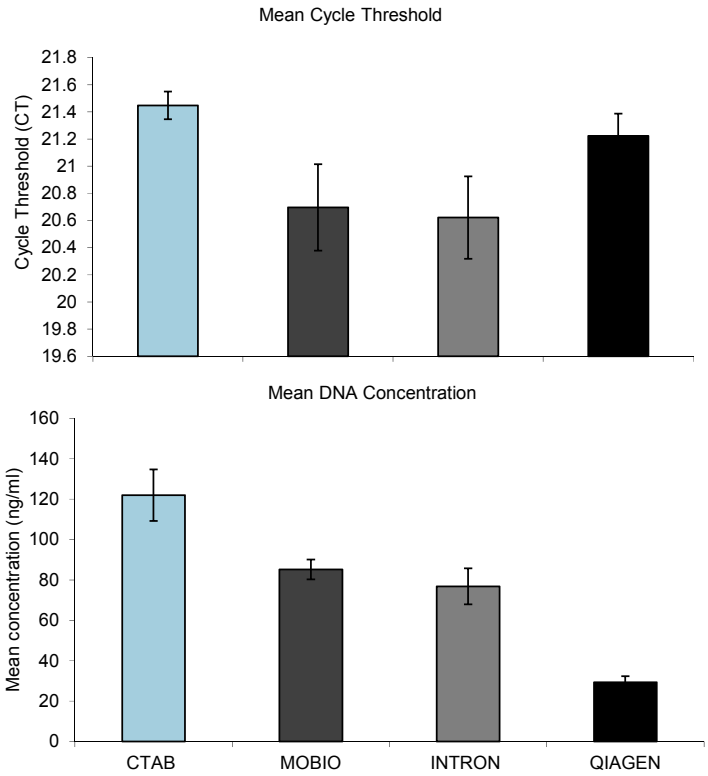

Figure 14 Performance of different DNA extraction kits. 'Mobio' and 'Intron' kits had similar CT values and DNA concentrations and were not statistically different.

4.5.1. Optimisation of qPCR assay for the detection of saxitoxin-producing dinoflagellates in seawater

The DNA extraction efficiency of cultured cells and field samples was assessed using four DNA extraction methods (Figure 14). The assay was optimised using various primer concentrations, annealing temperatures and magnesium concentrations. Standard curves were established using known concentrations of PCR product run against different strains of cultured *A. catenella* isolated from around New Zealand (Figure 15). The standard curves were used to determine the efficiency of the assay and gene copy number. The limit of detection of the qPCR assay was also determined.

4.5.2. Sampling and analysis of field samples

Semi-quantitative assays were run on natural sea water samples from Queen Charlotte Sound and the Bay of Plenty; collected during *A. catenella* blooms in these areas. Assays were also run that successfully detected stxA genes in *A. catenella* resting cysts in natural sediment samples from the Marlborough Sounds. The relationship between cell numbers estimated by microscopic counts and qPCR in natural samples was highly significant.

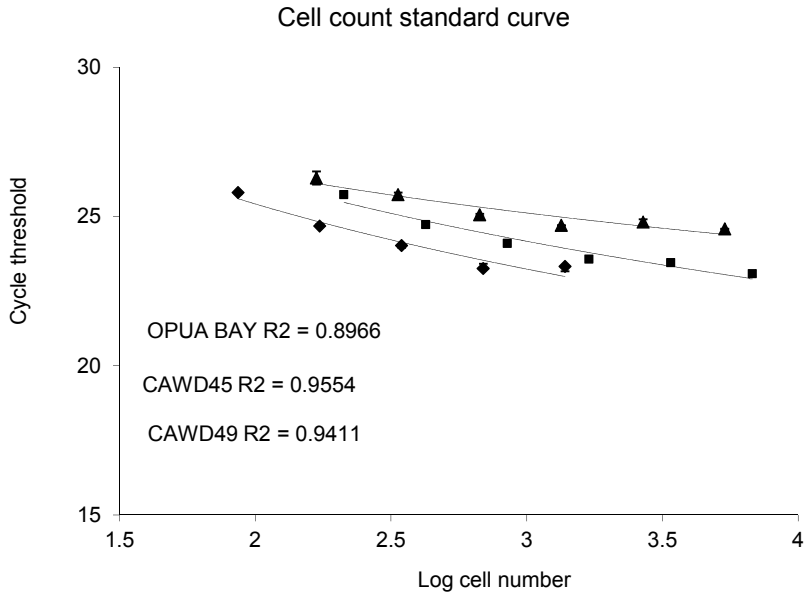

Figure 15 Regressions of cell numbers against qPCR cycle threshold using cultured cells from the Bay of Plenty (CAWD45 and CAWD49) and Opua Bay.

4.5.3. Significant outcomes

Comparison of the DNA extraction methods identified similar efficiency and DNA concentrations. However, the DNA extraction kit developed by Mo Bio was preferred as it is specifically designed for extracting DNA from soil samples. Our aim is to extract DNA from environmental samples and this kit is optimised for the removal of environmental PCR inhibitors (a common problem with environmental samples). This kit was also preferred because it uses a bead beater step which facilitates the rupture of thick walled *A. catenella* cells and cysts.

Using a culture of *A. catenella* cells from Tauranga (strain CAWD45 from the Cawthron micro-algae culture collection) we found we were able to reliably detect from 1 to 100,000 cells using the assay.

The mean copy number of the saxitoxin gene (sxtA4) per cell was calculated using three different cultured *A. catenella* strains. This was achieved by making a dilution series of known concentration of PCR product to calculate the number of saxitoxin gene copies in the cultured samples, and these were divided by the known number of cells in the original DNA extraction, to obtain copy number per cell. The results indicate that the mean copy number of sxtA4 gene in the three cultured samples ranged from 188-480 copies per cell. Compared to work on overseas *A. catenella* isolates (Murrary *et al*. 2011), the stxA gene copy number is slightly higher in the New Zealand strains.

These results lay the essential ground work required for the further development of a standard method, which will ultimately require evaluation by food safety regulators (MPI-Food Safety) as to whether it is suitable for use in the New Zealand shellfish biotoxin monitoring programme.

5. DISCUSSION AND CONCLUSIONS

There is good evidence that Opua Bay, Tory Channel, has a resident population of *A. catenella* and is the origin of annual blooms. The timing of development and demise of the bloom in 2012 was very similar to that of the previous year, and, because the bloom originates from the germination of benthic cysts it is likely that this timing will remain constant from year to year. The high dinoflagellate biomass that occurs in Opua Bay in summer probably relates to its proximity to the high nitrate environment of Tory Channel that is constantly fed with nutrient-rich upwelling water from Cook Strait.

The 2012 bloom did not grow to the very high cell densities seen in 2011, because a series of weather systems crossed the Sounds that brought strong winds in late

February and early March, stirring up the water column and resulting in suppression and dispersion of the bloom. A simple growth projection suggests that if this had not happened, cell concentrations equivalent to those of 2011 would have occurred in late March at the same time as the peak of the bloom in that year. A working hypothesis is that unless these high cell numbers are achieved in Opua Bay there is not a sufficiently large inoculum to initiate a bloom throughout the rest of the Sound, when the losses that probably occur when it enters the turbulent waters of Tory Channel and the generally nutrient depleted condition of the water column throughout the rest of the Sound (excluding Tory Channel) at this time of year, are taken into account. Only low numbers of *A. catenella* were seen outside the Onapua/Opua Inlet in 2012 and only low to trace levels of STX contamination were detected at routine monitoring sites.

Biological factors may also have played a role in restricting bloom development in Opua Bay in 2012. During the 2011 bloom there were very few other phytoplankton species present; in 2012 the non-toxic dinoflagellate *Akashiwo sanguinea* was also very numerous and simply through competition for light and nutrients may have played a role in supressing the growth of *A. catenella.*

A. catenella seems to be becoming more common in several regions. Blooms of *A. catenella* occurred from the Bay of Plenty to the Bay of Islands on the North Island east coast from January to March 2012. It was seen in samples from Manukau Harbour in January and recently (June 2012) in samples from Kaipara Harbour.

The cyst survey in February 2012 found no evidence that *A. catenella* has spread beyond Queen Charlotte Sound and the pattern of distribution in Queen Charlotte Sound was similar to that observed the previous year. Presumably, in 2012 cysts germinated throughout Queen Charlotte Sound, but their density was insufficient to initiate a bloom outside Onapua/Opua Inlet. Experiments have shown that nearly all cysts in surface sediments are viable and capable of producing populations of motile cells. The very high numbers of cysts in the Opua Bay sediments are undoubtedly the origin of the annual blooms but there was no evidence of a sudden explosion in cell numbers that might have resulted from a synchronised mass germination of cysts. It is probable that there is slow trickle of germination throughout spring and summer that leads to establishment of the planktonic population in late summer. Next year we will attempt to obtain estimates of *in situ* germination rates prior to and during the bloom period.

We still do not have a definite answer regarding the question of how long *A. catenella* has been resident in Opua Bay, but the sediment core analysis suggested that it may be much longer than we expected. To address this question we will need to obtain deeper, good quality cores (collected by SCUBA) and isotopically (^{210}Pb) date sediment layers. To accomplish this will be a research priority in the next year. If, in fact, *A. catenella* has been resident in Opua Bay for several decades, perhaps it is

only in recent years that it has become sufficiently established that it is able to form blooms that can extend beyond the confines of the Onapua/Opua Inlet.

In summary; annual *A. catenella* blooms in Queen Charlotte Sound are probably here to stay; the species is well established in the Onapua/Opua Inlet off Tory Channel. Whether it is firmly established in other regions of Queen Charlotte Sound as well is still uncertain although cysts exist in sediments throughout the sound. Annual blooms in late summer to autumn (February to April) can be expected in the future. We cannot be certain it will spread into other areas of the Sounds and Tasman and Golden Bays but, if it does the industry may have to manage production around annual region-wide closures of 2 to 3 months in late summer and autumn.

6. RESEARCH PRIORITIES

Research will continue on the shellfish toxin analysis using the Lawrence method to establish a sufficient body of validation data for publication in an appropriate international journal. If the validation exercise supports it, this will enable regulators to permit closure and opening decisions based on a level of ≥ or ≤ 0.8mg/kg STX equivalents on the screen test.

Research and validation of the STX receptor-binding assay for use as regulatory tool in New Zealand is going ahead at Cawthron Institute. It is likely that this assay will provide a more rapid and cost-effective means of protecting public health and the shellfish industry from STXs in the future.

A major emphasis of research will be to obtain environmental data over the 2013 bloom season in Tory Channel and the Onapua/Opua Inlet. This will enable us to more precisely identify the conditions under which the blooms are initiated, terminated and dispersed, to achieve the long term objective of developing a biophysical bloom simulation and dispersion model for Queen Charlotte Sound and the wider region. To the extent that funding will permit, this will include intensive field sampling and analysis between January and April 2013 and the installation of a variety of *in situ* monitoring equipment.

6.1. Sampling

- Cyst counts and isotope dating of sediment cores from Opua Bay.
- Estimation of *in situ* cyst germination rates. This will be challenging and will require the design of special equipment.
- Estimation of *in situ* growth rates.

- Experimental determination of temperature and salinity optima and nutrient assimilation dynamics of cultured Opua Bay *A. catenella* strains.
- Detailed sampling of the Tory Channel and the Onapua/Opua Inlet water column on a number of occasions over the bloom period, including CTD casts, nutrient analyses and phytoplankton biomass and composition.
- Installation of an ADCP current meter and weather station buoy in Onapua Bay over the bloom period.
- Placement of temperature loggers, sedimentation traps and mussels on the Opua Bay buoy.
- Repeat of the Sound-wide cyst survey including Port Underwood and outer Queen Charlotte Sound.
- Continue weekly phytoplankton analysis of the Opua Bay water column.

6.2. Modelling

If *A. catenella* spreads throughout the 'Top of the South', the best long-term option for managing and predicting the risk, severity and dynamics of future blooms of *A. catenella* is to obtain a thorough knowledge of its ecology within the local hydrographic and water chemistry context, and use these data in the formulation of a numerical bloom simulation model. This approach has been successfully applied in the Gulf of Maine, USA (http://www.ices.dk/products/CMdocs/CM-2010/N/N0110.pdf). During the bloom season, the Gulf of Maine Model (GMM) provides weekly predictions of bloom location and intensity based on weather forecasts. The Marlborough Sounds Bloom Simulation Model (MSBSM) would adapt the Gulf of Maine biological model and link it to a recently developed high resolution hydrodynamic model (Knight & Beamsley, 2012) that simulates water circulation within Marlborough Sounds under the influence of tides and local weather conditions. Ultimately, bloom simulations would be initiated and updated in real-time from field samples collected in the course of MSQP's routine monitoring. The GMM benefits from over a century of oceanographic research carried out in that region, whereas systematic observations of water column characteristics, nutrient and plankton dynamics barely exist for some important areas within the Marlborough Sounds. The systematic collection of synoptic water quality data over several years is critical to development of the MSBSM. The detailed observations that will be made over the next couple of years will define the parameters which control the growth and reproduction of *A. catenella* in the Sounds.

Dr Don Anderson (Woods Hole Oceanographic Institute, USA), the leader of the Gulf of Maine Alexandrium bloom ecology programme, and the chief modeller Dr Dennis McGillicuddy, have expressed their interest and willingness to collaborate (including the provision of the computer code) with the development of a numerical bloom simulation model for the Marlborough Sounds. This will bring the very best

international expertise available to assist with this and bring the benefits from the several millions of US dollars that have been invested in the Gulf of Maine model to date. To foster this collaboration we plan to apply for funding through MSI's International Mobility Fund USA for reciprocal visits between Cawthron Institute and the Woods Hole Oceanographic Institute over a period of three years to formulate and field trial the model.

7. ACKNOWLEDGEMENTS

Thanks to MSQP (Helen Smale, Noel McArthur) for carrying out the weekly sampling in Opua Bay, and for the use of the routine Queen Charlotte Sound shellfish toxicity and phytoplankton monitoring data. The weekly Opua Bay phytoplankton, shellfish toxin, and cyst analyses, were funded under Cawthron's Seafood Safety Programme (MSI contact CAWX0703). Ms Ashleigh Watts was funded through an MSI Undergraduate Internship and Mr Sam Webber was funded by a Bayer Boost Scholarship. Thanks to the New Zealand Meteorological Service for providing the weather maps and the Marlborough District Council for the Queen Charlotte Sound nutrient data.

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9. APPENDICES

Appendix 1 *Alexandrium catenella* counts from various locations on the North Island coast 2008 to 2012.

Alexandrium catenella **at Nth Island monitoring sites 2008-2012**					
Hauraki Gulf- Northland					
Location	**Sampling date**	**Cells/litre**	**Location**	**Sampling date**	**Cells/litre**
Bay of Islands, Kerikeri	1-Dec-08	100	Mangonui Wharf	2-Mar-09	1600
Bay of Islands, Kerikeri	22-Dec-08	200	Mangonui Wharf	9-Mar-09	3100
Bay of Islands, Kerikeri	29-Dec-08	700	Mangonui Wharf	11-May-09	500
Bay of Islands, Kerikeri	5-Jan-09	1400	Mangonui Wharf	10-Mar-10	500
Bay of Islands, Kerikeri	12-Jan-09	1300	Mangonui Wharf	15-Mar-10	400
Bay of Islands, Kerikeri	19-Jan-09	12000	Mangonui Wharf	22-Mar-10	500
Bay of Islands, Kerikeri	27-Jan-09	700	Mangonui Wharf	20-Apr-10	100
Bay of Islands, Kerikeri	2-Feb-09	300	Mangonui Wharf	17-Jan-11	400
Bay of Islands, Kerikeri	30-Mar-09	800	Mangonui Wharf	28-Feb-11	300
Bay of Islands, Kerikeri	9-Jun-09	100	Mangonui Wharf	9-Mar-11	600
Bay of Islands, Kerikeri	10-May-10	100	Mangonui Wharf	5-Sep-11	100
Bay of Islands, Kerikeri	31-May-10	100	Mangonui Wharf	9-Jan-12	400
Bay of Islands, Kerikeri	22-Jun-10	500	Mangonui Wharf	30-Jan-12	100
Bay of Islands, Kerikeri	13-Jul-10	600			
Bay of Islands, Kerikeri	24-May-11	600			
Patricks Point	2-Feb-09	100	Great Barrier Is. Port Fitzroy	6-Apr-09	100
Patricks Point	6-Apr-10	100	Great Barrier Is. Port Fitzroy	14-Apr-09	100
Patricks Point	11-May-10	100	Great Barrier Is. Port Fitzroy	27-Apr-09	2500
Patricks Point	30-Mar-11	300	Great Barrier Is. Port Fitzroy	28-Mar-12	6200
			Great Barrier Is. Port Fitzroy	2-Apr-12	12000
Bay of Islands, Tapeka Point	12-Jan-09	200	Marsden Point	7-Apr-09	100
Bay of Islands, Tapeka Point	19-Jan-09	5100	Marsden Point	21-Apr-09	100
Bay of Islands, Tapeka Point	27-Jan-09	200	Marsden Point	21-Sep-09	100
Bay of Islands, Tapeka Point	3-Mar-09	400	Marsden Point	8-May-12	300
Bay of Islands, Tapeka Point	23-Mar-09	3100	Marsden Point	2-Mar-10	800
Bay of Islands, Tapeka Point	30-Mar-09	200	Marsden Point	29-Mar-11	100
Bay of Islands, Tapeka Point	27-Apr-09	100	Marsden Point	23-May-11	100
Bay of Islands, Tapeka Point	15-Mar-10	200			
Bay of Islands, Tapeka Point	19-Apr-10	300			
Bay of Islands, Tapeka Point	26-Apr-10	1100			
Bay of Islands, Tapeka Point	10-May-10	800			
Bay of Islands, Tapeka Point	17-May-10	200			
Bay of Islands, Tapeka Point	21-Jun-10	300			
Bay of Islands, Tapeka Point	28-Jun-10	200			
Bay of Islands, Tapeka Point	7-Mar-11	100			
Bay of Islands, Tapeka Point	30-Mar-11	100			
Bay of Islands, Tapeka Point	28-Nov-11	100			
Bay of Islands, Tapeka Point	5-Mar-12	1000			
Bay of Islands, Tapeka Point	29-May-12	200			

Appendix 1 continued *Alexandrium catenella* counts from various locations on the North Island coast 2008 to 2012.

***Alexandrium catenella* at Nth Island monitoring sites 2008-2012**					
Bay of Plenty/East coast					
Location	**Sampling date**	**Cells/litre**	**Location**	**Sampling date**	**Cells/litre**
Tauranga Hbr	29-Dec-08	400	Te Kapa	6-Apr-10	300
Tauranga Hbr	15-Feb-09	100	Te Kapa	10-May-10	100
Tauranga Hbr	29-Mar-09	500	Te Kapa	9-Jan-12	300
Tauranga Hbr	24-May-09	100			
Tauranga Hbr	7-Feb-10	200	Bowentown Head Jetty	1-Mar-09	200
Tauranga Hbr	25-Apr-10	200	Bowentown Head Jetty	8-Mar-09	100
Tauranga Hbr	16-May-10	200	Bowentown Head Jetty	5-Apr-09	100
Tauranga Hbr	23-May-10	200	Bowentown Head Jetty	26-Apr-09	100
Tauranga Hbr	7-Jun-10	100	Bowentown Head Jetty	5-Sep-10	100
Tauranga Hbr	18-Jul-10	100	Bowentown Head Jetty	12-Jun-11	200
Tauranga Hbr	25-Jul-10	200	Bowentown Head Jetty	26-Jun-11	100
Tauranga Hbr	8-Aug-10	300	Bowentown Head Jetty	3-Jan-12	4900
Tauranga Hbr	19-Sep-10	100	Bowentown Head Jetty	8-Jan-12	600
Tauranga Hbr	31-Oct-10	200	Bowentown Head Jetty	15-Jan-12	3800
Tauranga Hbr	28-Nov-10	200	Bowentown Head Jetty	22-Jan-12	1400
Tauranga Hbr	19-Dec-10	1300	Bowentown Head Jetty	30-Jan-12	200
Tauranga Hbr	28-Dec-10	800	Bowentown Head Jetty	25-Mar-12	400
Tauranga Hbr	13-Feb-11	200			
Tauranga Hbr	12-Jun-11	300	Tairua	23-May-10	1000
Tauranga Hbr	6-Nov-11	100	Tairua	10-Jul-10	200
Tauranga Hbr	4-Dec-11	300	Tairua	13-Mar-11	100
Tauranga Hbr	11-Dec-11	400	Tairua	12-Jun-11	1800
Tauranga Hbr	18-Dec-11	200	Tairua	19-Jun-11	200
Tauranga Hbr	3-Jan-12	1400	Tairua	4-Nov-11	200
Tauranga Hbr	8-Jan-12	18000	Tairua	22-Jan-12	100
Tauranga Hbr	16-Jan-12	10000	Tairua	29-Jan-12	600
Tauranga Hbr	22-Jan-12	700	Tairua	12-Feb-12	100
Tauranga Hbr	30-Jan-12	100			
			Tolaga Bay	7-Sep-11	100
West coast					
Location	**Sampling date**	**Cells/litre**			
Manukau Harbour (Huia Bank)	23-Jan-12	200			
Kaipara Tinopai	1-May-10	200			
Kaipara Tinopai	23-Apr-12	100			
Kaipara Tinopai	5-Jun-12	1300			
Kaipara Tapora	22-Dec-09	100			

Appendix 2 Protocol for sorting of sediments and primuline staining of *Alexandrium catenella* cysts.

- Weigh original pottle containing sediment sample.
- Shake pottle, stir in order to ensure homogeneity
- Withdraw 2mL with blunt pipette, weigh, and transfer into 80mL beaker.
- Sonicate 2 minutes.
- Wash onto 80µm plankton sieve using tap water; catch with large 1800mL beaker.
- Wash thoroughly with tap water until 1800mL beaker is full.
- Pour beaker contents onto 20µm sieve, wash thoroughly.
- Repeat if the sample contains much coarse material.
- Wash back into same 80mL beaker using tap water from a spray bottle.
- Re-sonicate 30 seconds.
- Wash onto 20µm sieve, wash into 25mL measuring cylinder, and make up to 25mL.
- Shake vigorously and quickly sonicate ≥2 seconds. Withdraw 5mL sub-sample using 5mL pipette. Place in 15mL centrifuge tube.
- Add 100µL Glutaraldehyde, shake vigorously, and leave for at least 15minutes.
- Centrifuge for 25 minutes at 3000xg.
- Remove supernatant by withdrawing top 13mL using 10mL pipette. Remove last 1mL carefully using a Pasteur pipette. Hold centrifuge tube at 45°, being careful not to disturb the pellet.
- Add 15mL cold methanol. Shake vigorously. Place in cold room for ≥4 hours (less produces variable staining and variable counts).
- Centrifuge for 25 minutes at 3000xg.
- Remove supernatant in the same fashion as before.
- Add 15mL distilled water. Shake vigorously.
- Centrifuge for 25 minutes at 3000xg.
- Remove supernatant in the same fashion as before.
- Add 1mL primuline solution (0.08grams per 20mL).
- Leave to stain for 1 hour. Shake every 15 minutes.
- Centrifuge for 25 minutes at 3000xg.
- Remove supernatant in the same fashion as before.
- Add 15mL distilled water. Shake vigorously.
- Centrifuge for 25 minutes at 3000xg.
- Remove supernatant in the same fashion as before.

- Make up to 5mL with distilled water.
- Shake vigorously and quickly sonicate ≥2 seconds. Quickly withdraw 0.25mL and transfer into sterile Utermohl chamber. Wash out pipette tip.
- Add distilled water such that the utermohl chamber is ¾ full.
- Leave to settle for at least 20 minutes.
- Count under 100x magnification.

Appendix 3 A survey of *Alexandrium catenella* cyst numbers in the surface sediments (0-1cm) of Pelorus, Kenepuru and Queen Charlotte Sounds 15 to16 February 2012.

Location	Latitude (Sth)	Longitude (East)	*A. catenella* cysts/ml
Upper Kenepuru	41.1843	174.0521	0
Waitaria Bay	41.1753	174.0289	0
Portage Bay	41.1937	174.0193	0
Te Mahia Bay	41.2104	173.9668	0
Snapper Pt	41.2020	173.9377	0
Kenepuru Ent	41.2180	173.8704	0
Nydia Bay	41.1526	173.8328	0
Fairy Bay	41.1169	173.8585	0
Nth East Bay	41.0918	173.8894	0
Crail Bay	41.1162	173.9629	0
Clova Bay	41.1005	174.0298	0
Laverique Bay	41.0491	174.0418	0
West Beatrix Bay	41.0398	174.0020	0
Richmond Bay	41.0112	173.9617	0
Ketu Bay	40.9854	173.9826	0
Forsyth Bay	40.9952	174.0187	0
Cannon Point	40.9251	173.9755	0
Waitata Bay	40.9714	173.9308	0
Hallam Cove	41.0066	173.8181	0
Brightlands Bay	41.0563	173.8552	0
Torea Bay	41.2218	174.0316	0
Shakespeare Bay	41.2720	174.0013	50
Grove Arm	41.2663	173.9415	50
Onahau Bay	41.2395	173.9751	417
Lochmara Bay	41.2285	174.0001	50
Blackwood Bay	41.2076	174.0958	17
Ruakaka Bay	41.2046	174.1206	50
Opua Bay 1	41.2660	174.2024	1567
Opua Bay 2	41.2684	174.2042	2650
Opua Bay 3	41.2676	174.2073	3550
Onapua Bay	41.2635	174.1904	1433
Oyster Bay	41.2540	174.2545	50
Ngaruru Bay	41.2350	174.2266	17
Double Bay	41.2166	174.1893	17
Umuheke Bay	41.2143	174.2290	17
Otanarau Bay	41.1731	174.3197	500
East Bay	41.1562	174.3587	525