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Review of Tetrodotoxins in the Sea Slug *Pleurobranchaea maculata* and Coincidence of Dog Deaths along Auckland Beaches

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Review of Tetrodotoxins in the Sea Slug *Pleurobranchaea maculata* and Coincidence of Dog Deaths along Auckland Beaches

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1 Executive summary

This report shows that dog deaths that have occurred in the Auckland region since early July 2009 are due to poisoning by tetrodotoxin. A total of fourteen dogs with similar symptoms were poisoned after being on beaches in the Hauraki Gulf from Whangaparoa to Coromandel. It is likely that all the dogs came into contact with a sea slug (grey side-gilled sea slug - *Pleurobranchaea maculata*) containing tetrodotoxin at very high levels.

We know that tetrodotoxin is a potent neurotoxin and that 1-2 mg can kill a person. Sea slugs collected from Cheltenham and Narrow Neck Beaches on Auckland's North Shore contained up to 0.85 mg per gram and were found to contain toxin on their skin in high concentrations. Although tetrodotoxin has not previously been reported in New Zealand, or in sea slugs, the findings are alarming and there is potential for human poisoning. Death from tetrodotoxin is common worldwide through the consumption of contaminated food or from the bite of a tetrodotoxin carrying octopus.

We do not know if these sea slugs commonly contain tetrodotoxin. It may be that during 2009 there have been more sea slugs, more dogs or a higher concentration of tetrodotoxin in sea slugs than normal in the Auckland area. The poisonings may be due to a combination of some or all of these factors. Little is known about the source of the sea slug contamination, however, it may lie in the shallow sub-tidal crustose turf/benthic algal communities adjacent to these beaches. It may be that tetrodotoxin occurrence in sea slugs or other organisms is sporadic and limited in geographic extent and systematic surveys are needed to determine this.

Due to the potential for harm to people and the novelty of this event there are a number of short-term recommendations requiring urgent attention.

1. Erect signage to warn the public
2. Determine if other organisms that are potentially edible contain tetrodotoxin
3. Determine the national distribution of this toxic sea slug
4. Commence research into the sea slug through laboratory based studies
5. Provide testing services for potential clinical cases

Other recommendations include the need for a long term strategic look at establishing a framework for similar multi-agency investigations in future as it must be noted that this investigation was unusual because a toxin was quickly identified. In order for this to occur a national response policy needs to be established that facilitates the kind of co-operation demonstrated during this event with the potential for an investigation to become long term.

2 Objectives

The objectives of this report are to provide the following:

- A summary of investigations completed up to 27 August 2009 into dog deaths on beaches in Auckland, including evaluation of possible links to deaths of marine life during June and July 2009.
- A summary of toxin testing results from Cawthron.
- A review of current knowledge tetrodotoxin and the ecology of the sea slug *Pleurobranchaea maculata.*
- Interpretation of the event within the context of surrounding human environmental influences.
- Recommendations for future actions, and monitoring and surveillance over the short to long term.

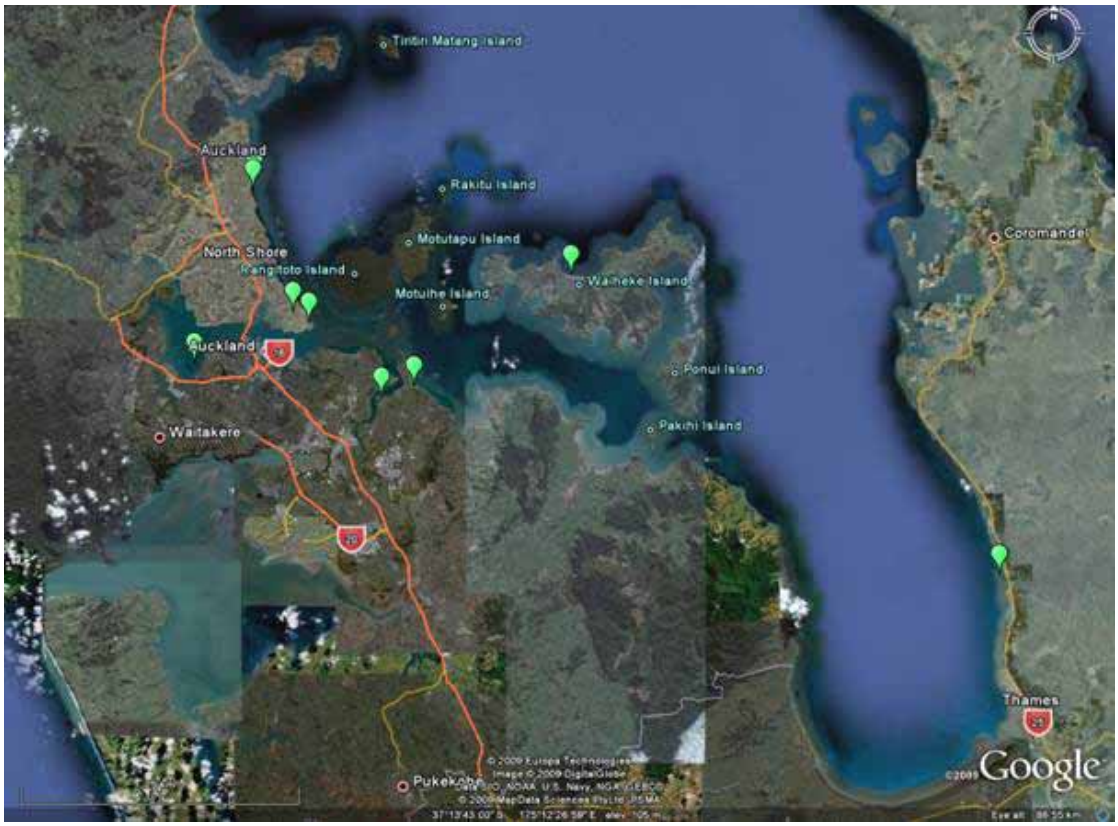
3 Description of problem

3.1 Notification of problem

Over 300 calls have been made to the Ministry of Agriculture and Forestry (MAF) free phone (0800 80 99 66) reporting incidences of suspected animal poisoning including dog, penguins, pilchards and dolphins. Forty-one calls related to dogs poisonings and investigation by MAF (National Centre for Disease Investigation) has resulted in 14 possible cases being defined. The location of these cases, plus four cases subsequently removed after further analysis, is detailed in Figure 1.

As a result of media reports into these animal deaths there has been significant public interest in the results of this investigation. All the agencies affected (Appendix A) by dog poisonings and the potential for harm to people in the area have received significant number of phone calls from the public. However, the calls by veterinarians to the MAF free phone initiated a formal response.

Figure 1. Locations of dog poisoning incidents (Green symbols).



Calls to the MAF free phone are assessed by staff at the National Centre for Disease Investigation and a cluster is noted based on their professional judgement. In this case

the rapid onset of vomiting, often before the dog had left the beach followed by the rapid onset of other symptoms was judged to be irregular.

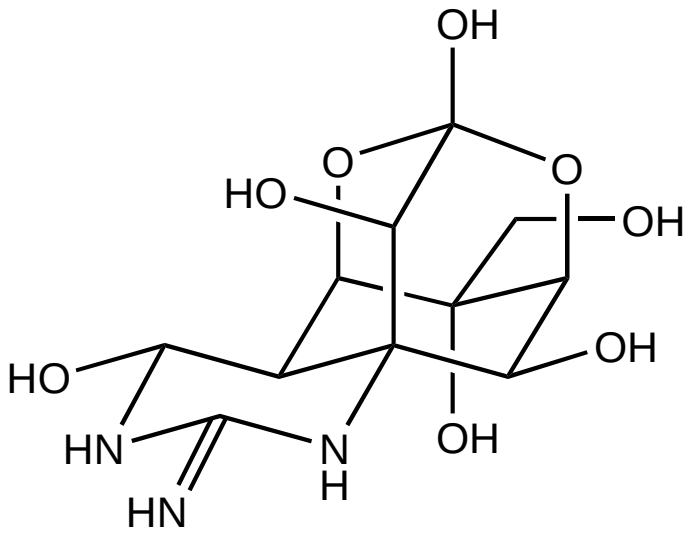
Cases as per MAF Biosecurity New Zealand (BNZ) were defined as: "The common symptoms of these dogs include vomiting, ataxia, bradycardia, and lethargy. The variable symptoms include salivation, dysphasia, diarrhoea, muscle fasciculation, seizures, respiratory failure, cardiac arrhythmia and death. The case definition is a dog that has been to a beach with one or more of the common symptoms with or without the variable symptoms reported to the MAF 0800 number in the period 14 July – 27 August 2009. The onset of clinical signs must be within 48 hours of being at a beach." The cases could not be definitively confirmed by toxicology as dogs that died had been disposed of before the investigation was officially commissioned. Similarly, blood and urine samples from recovered dogs were not available due to the time period that had elapsed and limitations in the detection limits of the clinical assay.

3.2 Identification the cause of the problem

Tetrodotoxin (TTX,

Figure 2) was identified by Cawthron in a composite sample of three sea slugs (*Pleurobranchaea maculata*) (Figure 3) collected from Narrow Neck beach on 7 August 2009. Tetrodotoxin was also found in the vomit of one dog that died soon after visiting Narrow Neck beach (Case 3, Appendix B).

Figure 2. Tetrodotoxin (TTX).



TTX is a potent neurotoxin known to occur in a wide range of marine organisms including puffer fish, xanthid crabs, newts, ribbon worms, blue ringed octopus, and others. TTX poisoning is very consistent with the case definition described above and has been divided into 4 grades based on severity of poisoning (Kaku *et al* 1995).

- Grade 1: Numbness around mouth, paresthesia, nausea

- Grade 2: Numbness of face, tongue and other areas, early motor paralysis and incoordination, slurred speech, reflexes normal
- Grade 3: Widespread paralysis, dyspnea, hypotension, fixed dilated pupils, patient still conscious
- Grade 4: Severe respiratory failure and hypoxia, hypotension, bradycardia, Cardiac dysrhythmias, patient may be unconscious, death due to respiratory failure

The levels of TTX found in samples of sea slug ranged from 91 to 850 mg/kg with a median of 385 mg/kg (n=12) refer to Table 1. The oral toxicity of TTX in dogs is not known, however the minimum lethal dose in humans is 1-2 mg (Yin *et al* 2005, Noguchi & Ebesu 2001). Thus a lethal dose may be as low as 0.1 mg of TTX for dogs (based on a small dog being one tenth of a human in size and assuming dogs are as sensitive as humans). Therefore, based on a median value of 0.385 mg/g it is possible a quarter of a gram of sea slug may be fatal to a dog and two grams for a human.

Other samples (mussels, oysters, pilchards, sponges, porcupine fish, sea cucumber, another species of sea slug and two other dog vomit samples) were also tested for TTX and all were negative down to 0.05 mg/kg.

Figure 3. Photos of sea slugs (*Pleurobranchaea maculata*) in captivity at Cawthron Institute. The slug pictured at right is laying an egg mass. These slugs were approximately 60 mm long.

3.3 Other organisms considered

One hundred and thirty-nine calls to the MAF free phone reported dead penguins washed up on beaches. These reports were over a much wider area (Northland to Bay of Plenty). Six penguins have been examined post mortem by the New Zealand Centre for Conservation Medicine and the results indicate these specimens died of starvation. Histology on two specimens did not indicate acute poisoning. There is no evidence that links the dog deaths to penguin deaths. It is not uncommon for penguins to wash up dead on New Zealand beaches.

Since 9 July 2009 there have also been eight dead common dolphins found in the Hauraki Gulf region. The results of post mortem analysis of six animals were not diagnostic and no cause of death could be determined. Testing of dolphin specimens was not considered necessary due to the very specific pathways of TTX transmission, the need to focus effort on the most likely vector species, and the risk of false negatives.

Reports of dead pilchards washing up on beaches around the Whangaparoa peninsula were received. The first report was around mid-July and the last report was 31 July 2009 at Long Bay. A similar mortality event occurred in the same region (and subsequently nationwide) in June 1995, which was attributed to pilchard herpes virus. MAF BNZ has commissioned specific tests for pilchard herpes virus and results are pending.

Reports of other fish species washing ashore dead have also been received around Whangaparoa (*e.g.* porcupine fish, snapper) but not high enough numbers to be considered significant.

3.4 Consideration of 1080 and brodifacoum

The Department of Conservation (DOC) initiated testing for brodifacoum in penguins, dolphins and dogs due to concerns that their pest control programme on Rangitoto and Motutapu Islands could be the cause of deaths in these species. Independent veterinary post-mortem, symptom presentation assessment and histology test results have all been negative to brodifacoum poisoning. Brodifacoum testing on the same dog vomit that contained TTX was negative. The discovery of TTX in this dog vomit removes any chance the death might have been from anything but TTX.

The post-mortem analysis of penguins by the New Zealand Centre for Conservation Medicine, showed no evidence of an acute poison as the possible cause. The analysis concluded that the birds were in poor body condition and that starvation was the likely cause of death. The post-mortem analysis of dolphins by Massey University did not reveal any evidence of brodifacoum poisoning. No 1080 or brodifacoum testing of pilchards was completed; however, these mortalities were in a location remote to recent pest control activities, they involved multiple locations reported from around Whangaparoa over a period of at least two weeks and were similar to previous events

reported in the 1990s, whereas you would expect a point source of contamination to produce a single event and affect multiple species.

DOC concludes that the application of brodifacoum to remove rodents from Rangitoto and Motutapu Islands had no relationship to the co-incidental death of dogs, pilchards, dolphins and penguins.

4 Affected agencies

Nineteen agencies were involved in the Joint Steering Group or the Technical Advisory Group (TAG). The agencies and nominated representatives are listed in Appendix A. Members of the public and businesses involved with the sea, such as tourist operators, have also been impacted by this event.

A brief telephone survey was conducted by Cawthron to see how a sub-section of affected agencies had initially become involved, what the key role of their agency had been and what information they found most useful from the steering group or the TAG. Some general trends emerged and some of the suggestions have been included as recommendations in this report.

In general, most organisations became involved due to a responsibility to investigate unusual events; for example, MAF BNZ to determine if it might be a new disease. Other organisations initially became involved to assist as best they could, Cawthron became involved as they had relevant skills to add to the existing information. The involvement of Auckland Regional Council (ARC) is perhaps the most interesting as the emerging issue appeared to be no more that a series of random, natural events that occur episodically in the Hauraki Gulf. However ARC agreed that a single coordinator was appropriate and formally commenced this role on 10 August 2009.

While it was not always clear why an agency had become involved, or taken on the role they did, it was interesting that each agency was very clear what their role had been and how they had contributed. MAF BNZ providing veterinary advice, New Zealand Food Safety Authority (NZFSA) providing advice on food, DOC fieldwork and assessment, Auckland Regional Public Health Service (ARPHS) providing health information to the public *etc*.

The most useful information generated was of a technical nature, narrowing the list of potential toxins based on the dog death case data and finding toxic sea slugs and TTX. The reason this information was useful was that the uncertainty had been reduced and constructive management and mitigation strategies could be initiated.

There was general agreement that the MAF free phone number (0800 809966) was very useful; mostly in aiding identification of a cluster of related events and then allowing links to other events to be tracked and ruled in or out as new information came to hand. DOC and ARPHS also have public phone lines and there seemed to be a clear distinction between events involving human illness and animal illness, but less clear distinction when it was an animal if it was the DOC or MAF free phone number that needed calling. MAF were also keen to emphasis the fact that calls to their number should be for unusual events. Some streamlining of this system between agencies seems desirable and while it may be the responsibility of one agency to run the 0800 number the other agencies need to ensure their area of expertise is well known to the 0800 call centre agency.

Finally it was noted that this event may not have even been recognised for what it was if it had occurred outside of Auckland. The veterinarians of Auckland are to be praised

for diligent notification to MAF and the close working relationship between the agencies in Auckland was evident.

5 Actions taken to investigate the problem

A wide range of actions were taken at various times throughout the investigation and the significant findings were well summarised by regular situation reports and in a final report on the testing performed at Cawthron. A full summary of those reports can be found in Appendices C and E. However, the brief time-line of actions followed are described here.

5.1 Summary of events, based on Situation Reports (Appendix C)

7 August 2009

- MAFBNZ commenced a surveillance and casing project for dog- and penguin-related calls to the MAF free phone and as part of this asked the National Poisons Centre to create a short list of potential causes.
- Testing and the rapid onset of symptoms eliminated brodifacoum and other pest poisons as potential causes; and testing for toxic algae found no evidence of these.
- Toxins and particularly neurotoxins were correctly identified as a likely cause and initially a link between dog, dolphin, penguin and pilchard deaths could not be ruled out.
- North Shore City Council erected signs on beaches. Water samples were taken from 18 beaches on the North Shore and analysed for faecal bacteria and toxic algae. Nothing of concern was identified.

13 August 2009

- A sea slug (*Pleurobranchaea maculata*) was identified from samples collected by Cawthron at Narrow Neck beach and TTX was provisionally identified as the toxin from a composite sample of three individuals.
- Evidence of bloom-level chlorophyll was observed by National Institute of Water and Atmospheric Research (NIWA) in the first two weeks of July; however, there was no other evidence to support the hypothesis that a pelagic algal toxin was responsible; particularly as regular monitoring by the NZFSA in the Hauraki Gulf revealed nothing of concern.
- Post mortem analysis of penguins by New Zealand Centre for Conservation Medicine indicated they had died of starvation.
- Auckland City Council joined North Shore City Council in erecting warning signs on beaches. Significant numbers of *P. maculata* were only found at Narrow Neck beach and one at Cheltenham despite extensive searches at seven beaches on

the North Shore and two in Tamaki/East Auckland. Further surveys of the Firth of Thames were undertaken by NIWA on behalf of Environment Waikato, no sea slugs were found.

21 August 2009

- More biological samples were collected and tested for TTX by Cawthron. Results showed only sea slugs and one dog vomit sample from Narrow Neck and Cheltenham beaches contained TTX.
- Surveillance was ongoing and reports from Long Bay and Stanmore Bay reported no slugs were found. No sea slugs were found on Manukau Eastern beaches following an extensive search.
- MAF BNZ continued to record reports of dead animals and sick dogs. From a total of 41 reported dog incidents 18 were considered possible cases.
- Information about the sea slug, *P. maculata,* and TTX, was compiled by Cawthron scientists and advice sought from Dr Richard Wilan, Curator of Molluscs at the Northern Territories Museum, Darwin Australia and from Auckland Museum.

5.2 Summary of ARC surveys

A number of comprehensive surveys were carried out by ARC during the period 12-28 August 2009 focusing on Narrow Neck and Cheltenham beaches as the collection of *P. maculata* specimens for further testing was considered a high priority. On 12 August 2009 several teams were deployed at Mount Wellington war memorial reserve, Eastern Beach, Long Bay, Brown's Bay, Milford Beach, Castor Bay, Takapuna Beach, Narrow Neck Beach and Cheltenham Beach. *Pleurobranchaea maculata* was only found at Narrow Neck (150-200 sea slugs) and Cheltenham (one sea slug). Only four larger individuals were found alive and most of the specimens were dead when collected. Other surveys were conducted on 17, 19, 21, 27 and 28 August 2009 at Narrow Neck and Cheltenham beaches. During these surveys between four and 24 specimens were found. The specimens found were mostly alive, and those tested by Cawthron contained TTX at levels consistent with the initial sample. Other beaches sampled were Long Bay and Stanmore Bay on 19 August 2009, but no sea slugs were found. On 28 August 2009 Takapuna, Milford and St Leonards Beaches were searched and no slugs were found.

5.3 Summary of Cawthron Report 1652 – (Appendix E)

Extensive sampling and testing was completed by Cawthron on a range of samples from 13 August – 1 September 2009 to look for a potential toxin as the cause of the dog deaths. No potential toxic algae were identified in any of the samples of dog stomach contents, dog vomit or seawater. A series of samples was tested for toxicity

using mouse bioassay and a sea slug *P. maculata* sample returned a strongly positive result. Subsequent work to identify the toxin focused on the sea slug sample and, as a result, TTX was identified in the sea slug sample.

Tetrodotoxin was confirmed in the initial sea slug sample by LC-MS. A LC-MS method of analysis was set up using authentic TTX and several known analogues of TTX were added to the method based on their known mass but no positive material was available to confirm their detection. When TTX was found it was generally at high levels and minor amounts of 11-nor TTX were also detected but not confirmed. The daughter ion spectra of authentic TTX and TTX from the initial sea slug were shown to be identical and ion ratios based on MRM channels were also consistent with finding TTX in sea slugs and dog vomit. In addition the HPLC retention time of authentic TTX and TTX from the sea slug and dog vomit samples matched very well (well within normal laboratory variation).

Confirmation of TTX in one dog vomit sample (Case 3, appendix B) provided a critical link between the dog deaths and the discovery of TTX in toxic slugs.

After confirmation of the identity of the toxin LC-MS was used to quantify the amount of TTX present in samples. Initial results were incorrect due to the calibration standard used. Corrected data is presented in Table 1.

Once the quantitative method was validated with a calibrated standard a single extract from the sea slug was tested by LC-MS and mouse bioassay. The extract was more toxic than expected based on the measured TTX content but no other acute toxin was suspected of being present. Some further checks are warranted but the most likely situation is that no other toxin is present.

Finally samples were tested for TTX levels, and the results are summarised in Table 1.

Table 1. Tetdrotoxin concentrations in samples by LC-MS.

Location	Date Sampled	Description	TTX mg/kg*
Narrow Neck Beach	7 August 2009	*Pleurobranchaea maculata* – initial sample of three sea slugs	380
Narrow Neck Beach	9 July 2009	Dog vomit – case 3, appendix B	18
Cheltenham Beach	12 August 2009	*P. maculata*	140
Narrow Neck Beach	12 August 2009	*P. maculata* – dissected	670
Cheltenham Beach	17 August 2009	*P. maculata* – dissected	91
Narrow Neck Beach	17 August 2009	*P. maculata* – dissected	450
Narrow Neck Beach	17 August 2009	*P. maculata* –dissected	350
Narrow Neck Beach	17 August 2009	*P. maculata* –dissected	320

Location	Date Sampled	Description	TTX mg/kg*
Nelson	18 August 2009	*P. maculata* –dissected	0.45
Narrow Neck Beach - south	21 August 2009	*P. maculata*	410
Between Cheltenham and Narrow Neck Beaches	21 August 2009	*P. maculata*	830
Narrow Neck Beach - mid	21 August 2009	*P. maculata*	850
Narrow Neck Beach - north	21 August 2009	*P. maculata* - large	240
Narrow Neck Beach - north	21 August 2009	*P. maculata* - small	390

* Wet weight, total of all tested tissues if dissected.

A large number of other samples were also tested for TTX and all were negative (<0.05 mg/kg). Samples collected from Cheltenham or Narrow Neck Beaches and tested were: Greenshell™ mussels, marine sponges, dog vomit (two other cases from the list in Appendix B) an algal mat, another species of sea slug (*Onchidella nigricans*) and limpets (*Cellana radians*). The dog vomit samples that tested negative were most likely from cases 1 and 10, Appendix B, and were not obviously vomit. It is likely that these samples were not initial vomit samples or they were material sampled from near a vomit site and appeared to be sand or bark chips.

Following up on specific enquiries several more samples were also tested for TTX and all were negative (<0.05 mg/kg). Porcupine fish (*Tragulichthys jaculiferus*), oysters (*Crassotrea gigas*) and pilchards (*Sardinops sagax*) from Long Bay were investigated after a report of someone becoming ill from the consumption of oysters but no TTX was found, nor evidence of other usual toxins or bacterial contamination. Pilchards from Orewa were gutted and the gut contents tested for TTX, none was found. A dog that died suspiciously at Stanmore Bay was tested (stomach contents and liver) and no TTX found, this dog was later determined to have died of cancer (MAF BNZ data).

A single specimen of *P. maculata* was retrieved from Nelson. Tetrodotoxin was detected but at very low levels (Table 1). As this was the only specimen available there was no opportunity to confirm this result, without replication we can only conclude that it is probable that *P. maculata* from other areas may also contain TTX. This is an area requiring further investigation.

Dissection of the sea slug samples into internal organs (mouth, digestive tract, gonad and other internal organs) and external organs (including skin and the remaining tissues) was completed to try and test if *P. maculata* might be acquiring TTX from its diet. The results varied greatly, with 11% to 85% of the total TTX being detected in the external organs. When dissection was performed on fresh samples the results indicated that most of the toxin is located externally (61%, 84%, 85%). However only

three samples were tested in this way and one of the samples was the Nelson specimen.

Sea slugs collected from Narrow Neck Beach on 17 August 2009 were received at Cawthron's Nelson laboratory. Five specimens were live and one dead. The dead specimen and one of the live specimens were tested immediately (after dissection) and the total levels of TTX were 450 (dead) and 350 (live) mg/kg. The remaining four specimens were transferred to an aquarium where they appeared to thrive. One specimen was removed and tested after 72 hours, the total level of TTX was 320 mg/kg.

6 Summary of toxin results

Symptoms from 14 cases of sick dogs were consistent with a poisoning event. Cawthron results show that one case (Case 3, Appendix B) is definitely linked to TTX poisoning; most likely from the ingestion of a sea slug. Inter-tidal sampling for toxic sea slugs (*Pleurobranchaea maculata*) has so far found them only at Narrow Neck and Cheltenham Beaches. Samples of the same species of sea slug from Nelson were found to contain TTX at levels approximately one thousand times less than sea slugs from Narrow Neck and Cheltenham Beaches. Tetrodotoxin levels remained very high in sea slugs transferred to an aquarium environment for 72 hours. The amounts found in the sea slugs are approximately half the highest ever recorded for a whole organism.

7 Review of TTX in *Pleurobranchaea maculata*

7.1 Tetrodotoxin summary

- Tetrodotoxin is a sodium channel blocking neurotoxin
- Tetrodotoxin is wide-spread in marine organisms (Table 2).
- Bacteria are known producers of TTX; most often bacteria of the genus *Vibrio.*
- Bacteria producing TTX may remain in organisms in a symbiotic relationship (Daly 2004).
- Symbiotic bacteria have been found in other sea slugs (Klussmann-Kolb & Brodie 1999; Paul *et al.* 2007; Piel 2009); although the link with TTX is not certain.
- The source of the sea slug contamination may lie in the shallow sub-tidal sediments and crustose turf/benthic algal communities.
- Consumers of bacteria, like nematodes, copepods and arrow worms are known to have extremely high levels of TTX (Kogure *et al.* 1996).
- High concentrations of TTX further up the food-chain may be due to bio-accumulation of the toxin (Kogure *et al.* 1996).
- TTX accumulation in animals may be used for defence or offence purposes.
- The spatial and temporal extent of high concentrations of TTX in organisms is largely unknown but has been reported as highly variable.
- Transient immunity of restricted populations may have lead to the accumulation of unusually high concentrations of TTX in *P. maculata.*
- Consumption of organisms containing TTX is known to kill cats, dogs and humans.

7.2 Tetrodotoxin (TTX)

Cawthron scientists identified the toxin within beach cast *P. maculata* as TTX and an extensive literature search was done to give background to the toxin and to provide biological and ecological context to the issue.

Tetrodotoxin has been identified in a remarkably wide range of marine, freshwater and terrestrial vertebrates and invertebrates and is present in at least 10 metazoan phyla (Table 2). Tetrodotoxin exerts its effect by having a very high affinity for a receptor on the sodium channels in the membranes of motor neurons. Attachment of TTX to the sodium channel receptor blocks the transmission of nerve impulses, resulting in

muscular paralysis. Saxitoxins (STXs) produced by planktonic dinoflagellates have the same effect.

Tetrodotoxin has been documented in at least 14 species of shelled gastropods, some of which have been associated with human fatalities (Appendix D). The finding of TTX in *P. maculata* on Auckland beaches is the first time it has been discovered in a soft-bodied sea slug species. The levels of TTX (up to 4,250 MU/g; 1mg TTX = 5,000 MU) found in *P. maculata* are high in comparison to levels observed in vertebrates but less significant when compared with invertebrates. Levels in puffer fish liver are on the order of 300-900 MU/g (Miyazawa & Noguchi 2001). The highest records we have been able to locate are 10,361 MU/g in the marine snail *Nassarius glans* and 13,000 MU/g in the ribbon worm *Cephalotrhrix linearis*.

There is a large volume of literature on tetrodotoxin in various organisms (summarised in Appendix D). However, there are various aspects of the phenomenon that remain uncertain. Most importantly, **although marine bacteria are implicated as the primary source, in many cases "the exact origin of TTX in the food chain remains unknown" (Noguchi & Arakawa 2008).** Although it is unclear from what source animals like puffer-fish and carnivorous gastropods acquire such high concentrations of TTX in their tissues, there is very strong evidence that it is obtained through food chain transmission and concentration. This is an area requiring further investigation.

TTX accumulation in animals appears to have several roles. In some it provides a defensive mechanism, whereby high concentrations in the skin, skin secretions and other organs such as the liver (*e.g.* puffer fish, newts) deter predators, and in others it plays an offensive role in prey capture (*e.g.* blue ringed octopus, arrow-worms). Because TTX is often found in high concentrations in the ovaries and eggs of some species (*e.g.* horseshoe crab, flatworms), it is also believed to have role in the protection of eggs and embryos/larvae from predation.

Table 2. A selection of marine animals known to contain tetrodotoxin (TTX)

Organism	Species name	Site of TTX accumulation	References
Puffer-fishes	*Takifigu vermicularis* and at least 21 other puffer-fish species	Ovaries, liver, skin	Mahmud *et al.* 2003 Wu *et al.* 2005. Lee *et al.* 2007 Bentur *et al.* 2008 Nunez-Vazquez *et al.* 2000
Gobies	*Yongeichthys criniger*	Skin viscera muscle, testis	Noguchi and Hashimoto 1973
Frogs & toads	*Brachycephalus ephippium* *B. nodoterga* *B. perni*	Skin and liver, ovaries	Pires *et al.* 2005
Flatworms	*Planocera multitentaculata*	Very high TTX levels (10,700 MU/g) in eggs	Miyazawa *et al.* 1986
Ribbon worms	*Cephalothrix linearis*	TTX highly concentrated in proboscis and protective mucous	Ali *et al.* 1990
Arrow worms	*Parasagitta elegans*	Carnivorous planktonic may use TTX to paralyze prey	Thuesen *et al.* 1988
Copepods	*Pseudocaligus fugu*	Puffer-fish parasite feeds on TTX containing skin	Ikeda *et al.* 2006.
Blue-ringed octopuses	*Hapalochaena fasciata* *H. lunulata*	Salivary glands, mantel, arms, digestive gland, testes, ovaries ink.	Williams and Caldwell, 2009
Horse shoe crabs	*Carcinoscorpius rotundata*	Flesh, eggs	Fusitani *et al.* 1982
Xanthid crabs	*Atergatis floridus* *A. germaini* *Lophozozymus pictor*	Muscle, gills, digestive gland	Noguchi *et al.* 1983 Saito *et al.* 2006
Newts	*Taricha granulose* *T. torosa* *Cynops pyrrhogaster*	Skin liver gonads, secretory glands in the skin	Lehman *et al.* 2004 Tsuruda *et al.* 2002 Mosher *et al.* 1965
Starfish	*Astropecten scoparius*	Gut contents, TTX probably acquired from preying on a toxic gastropod	Lin and Hwang, 2001. Lin and Hwang 2001

Tetrodotoxin has been detected in bacteria associated with marine and freshwater flora and fauna and sediments and this is a likely source of the toxin. Surprisingly, there are few accounts in the literature of attempts to track the source of the contamination by systematic sampling and analysis of benthic communities. The Hauraki Gulf/*P. maculata* event provides an excellent opportunity to resolve this issue

and every effort should be made to do this. The key to the source of the sea slug contamination may lie in the shallow sub-tidal crustose turf/benthic algal communities.

Tetrodotoxin-producing bacteria have been isolated from the gut and tissue of various species, but the role of these bacterial in the production of TTX within animal via symbiotic associations is contentious. Endogenous (produced within) production of TTX in terrestrial amphibians has been suggested but is as yet unproven.

There are some reports in the literature suggesting that TTX occurrence in gastropods is sporadic and typically limited in geographic extent. Edible shellfish in particular areas have poisoned people when there was no previous history of them doing so, and surveys have shown toxic populations of gastropods to be confined to discrete locations (*e.g.* Yasumoto *et al.* 1981). It is possible that this may also be the case with *P. maculata* in the Hauraki Gulf, and systematic surveys are needed to determine this.

Finally, TTX may play an important role in the ecology of *P. maculata* through the protection of itself and its progeny from predation and these creatures may always contain some level of these toxins. However, in other species (*e.g.* clams, garter snakes) it is known that small genetic changes can result in the acquisition of very high tolerance to sodium channel blocking agents such as TTX and STX. This can result in the transient immunity of restricted populations that leads to their ability to accumulate unusually high concentrations of these compounds in their bodies. Therefore, it is possible that selective pressure brought about by an unusual abundance of TTX in the habitat of *P. maculata* has resulted in a similar situation, causing these animals to contain extraordinarily high levels of toxin at this time. Ongoing monitoring and molecular analysis could determine whether these factors are involved in this phenomenon.

7.3 Ecology *of Pleurobranchaea maculata*

Pleurobranchaea. maculata is a carnivorous scavenger that is widely distributed in shallow subtidal areas around New Zealand, but is also found in south-eastern Australia, China, Sri Lanka and Japan (Gibson 2003). As with many opisthobranchs, *P. maculata* can be difficult to find and is highly cryptic, but it can be observed out in the open laying long white tubular egg sacs (Figure 3) during spring. It is possible that specimens of *P. maculata* that contain TTX at levels high enough to cause the poisoning of a dog at Narrow Neck beach, North Shore may be found within the area from Whangaparoa to North of Thames and possibly beyond that given it's New Zealand wide distribution. However, this is an area warranting further investigation before more definitive statements can be made.

At this stage we are unable to provide any robust evidence as to whether this is unique to the area or if this is a characteristic of these slugs on a wider geographical scale. It is possible, however, that there is a high level of spatial and temporal variability in the extent to which *P. maculata* accumulate the toxin; which in turn will depend on the availability of different food sources and perhaps factors like season and reproductive cycles. *Pleurobranchaea maculata* are highly opportunistic carnivores and scavenge on a range of invertebrate organisms (sea anemones, marine worms, other

molluscs); hence, the occurrence of high TTX within sea slugs in a given area could simply be due to an increase in a single food-source that accumulates TTX, and that this food-source is not consumed in great numbers by other organisms in the food web.

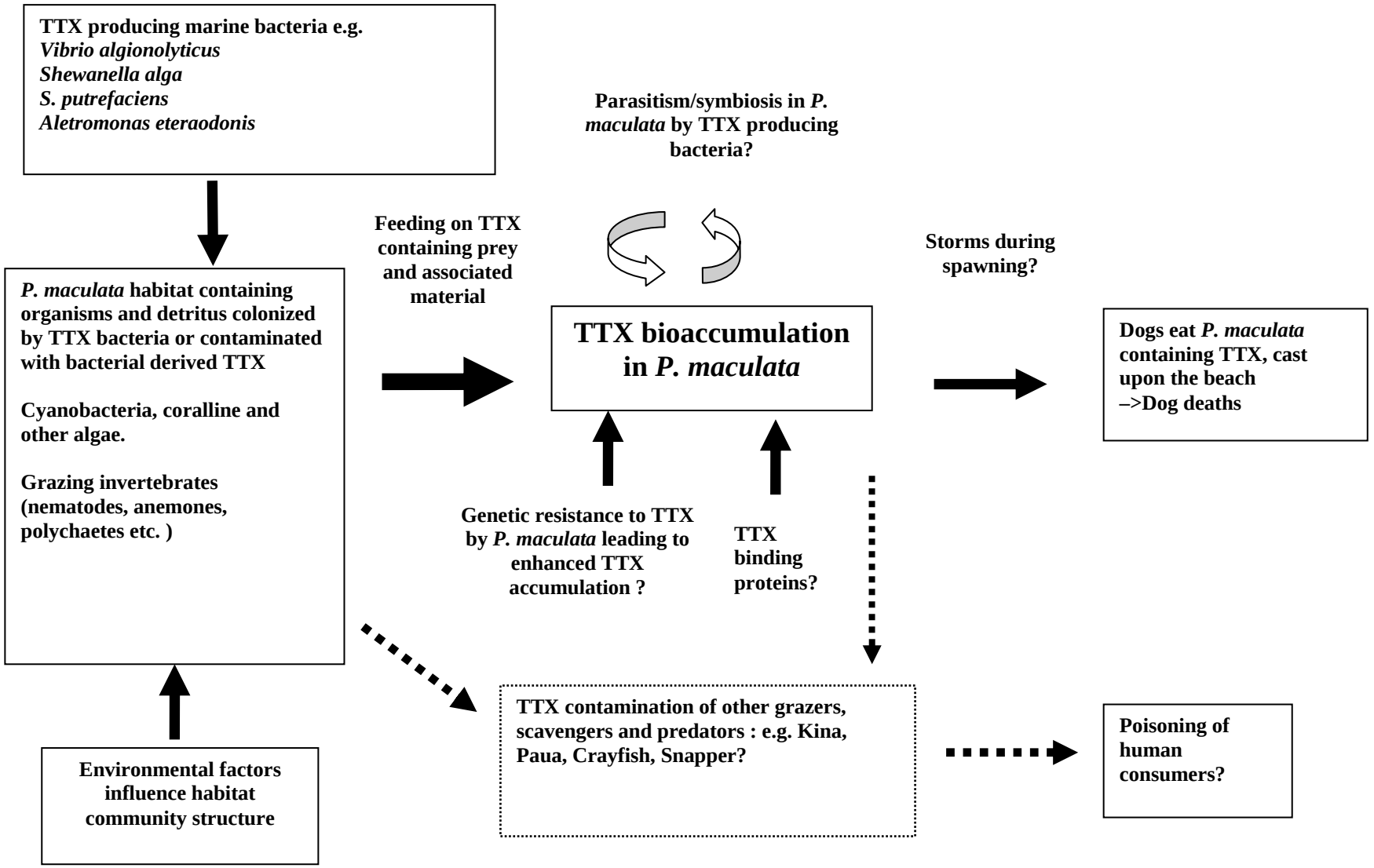
An interesting observation that has not been confirmed through subsequent experimentation is that TTX can apparently act as an attractant to puffer fish and starfish (Saito *et al.* 2000; Saito *et al.* 2003). Attraction to a TTX source within the benthic habitat or use of TTX as a pheromone could also explain the high concentration of toxic *P. maculata* off the Auckland beaches.

Possible mechanisms involved in tetrodotoxin contamination of *P. maculata* and poisoning of dogs in the Hauraki Gulf area are summarised in a diagram below (Figure 4). As yet, despite some reports of illness and follow-up testing, human illness related to TTX poisoning has not been confirmed in New Zealand.

Summary of *Pleurobranchaea maculata* (Quoy & Gaimard 1832) ecology:

- It is found in New Zealand, south-eastern Australia, China, Sri Lanka and Japan (Willan 1983; Gibson 2003).
- It is a carnivorous sea slug that feeds opportunistically, often on sea-anemones like *Actinia tenebrosa* (Ottaway 1977).
- It produces long cylindrical egg masses in the intertidal during early spring (Willan 1983; Rudman 1999).
- It is most commonly observed in the open during breeding season, but at other times can be found in and around rocky shores in sheltered harbours and bays (Willan 1983).
- It may be susceptible to stranding during breeding season when it is out in the open.
- It is highly cryptic and can be difficult to find.
- It has long been known to have a highly acidic mantle (pH2) (Willan pers. com.).

Figure 4 Possible mechanisms involved in tetrodotoxin contamination of *Pleurobranchaea maculata* and poisoning of dogs.



8 Human influences in relation to TTX

There has been speculation that the event of highly toxic *P. maculata* on Auckland beaches is linked to human impacts on the coastal environment, such as those associated with surrounding land uses (*e.g.* dairy farming) and subsequent inputs of land-derived contaminants. Agricultural runoff results in increased loading of nutrients and faecal contaminants to estuaries and coastal waters; storm-water runoff from urbanised catchments also leads to loading of contaminants such as metals and organic chemicals. The documented ecological effects of these contaminant inputs on the wider ecosystem are extensive. Including, nutrient enrichment-type effects such as increased production of phytoplankton and benthic macroalgae (*e.g.* Smith *et al.* 1999), degradation of shellfish resources due to faecal contamination, and adverse effects (including sub-lethal effects) on soft sediment organisms and communities associated with elevated trace metal and organic chemical concentrations (see review by Grant & Hay 2003).

The above types of human effects are not related to the bioaccumulation of TTX within *P. maculata*; TTX naturally occurs in the environment and many types of organisms concentrate the toxin in their tissues independent of human influences. What appears to be uncommon in this case is the co-occurrence of higher numbers of slugs on the beaches compared to previous years; most likely due to wave and tide conditions combining with annual breeding in the subtidal and intertidal zones leading to *P. maculata* becoming beach-cast, and oral contact with beach-cast *P. maculata* by dogs walking on the beach.

A possible link with land use impacts is through the food chain; for instance, in a scenario where human activities somehow influenced the abundance of a particular prey source that contains TTX, which in turn results in the accumulation of the toxin in the *P. maculata* that become beach-cast. However, this seems unlikely when we consider the episodic nature of the event, the range of wider ecosystem factors that can influence organism populations, and the extent to which the area is impacted by humans.

Long-term monitoring of water and shellfish quality suggests that water quality conditions near the affected beaches are well within the range of other estuaries and coastal areas around the country. Routine water quality monitoring results at Hauraki Gulf monitoring sites (*e.g.* Chelsea Wharf, Browns Bay, No.7 Buoy) rank as "good" or "very good" with respect to nutrient levels and additional water quality parameters (Scarsbrook 2008). The 2008 Hauraki Gulf State of the Environment report also indicates that the water quality conditions in the area haven't "worsened" through time and in fact some water quality indicators (*e.g.* nutrient and faecal bacteria levels) have improved in recent years (Hauraki Gulf Forum 2008). Accumulation of metals and organic contaminants in sediments within Auckland's developed harbours and poorly flushed estuaries remain a potential issue for soft sediment organisms such as shellfish (cockles) that inhabit these areas (Grant & Hay 2003). However, it is noted that levels of these contaminants in Auckland shellfish are considered low by international standards (Kelly 2007) and that the ecological effects of metals and organic chemicals within sediments is far removed from the processes affecting the accumulation of naturally occurring TTX within organisms such as *P. maculata*.

We also note that there are a number of aquaculture farms in the Hauraki Gulf and nothing out of the ordinary has been detected through the routine shellfish monitoring programme. Routine phytoplankton monitoring over the past ten years in the Hauraki Gulf Marine Park area, as part of the Marine Biotoxin Monitoring Programme, has on occasion detected the presence

of bloom-forming algae that produce toxins other than TTX. Continued monitoring has not detected harmful algal blooms within the region over the past three months (Appendix C).

The above observations collectively suggest that the presence of *P. maculata* with high levels of TTX is not linked to a 'wider ecosystem' problem. Furthermore, if we consider that water quality in the area is only moderately affected by the surrounding environment and that these effects haven't markedly changed in recent years, it appears highly unlikely that human effects have been the factor driving the episodic occurrence of highly toxic sea slugs on beaches.

9 Recommendations

There are some very important areas of further investigation because:

- We cannot rule out the potential for other marine species to contain TTX.
- We cannot rule out that sea slugs in other parts of New Zealand contain TTX.

We suggest immediate, medium and long-term actions below to address these issues (Sections 8.1 – 8.3).

Further recommendations of this report include:

- A need for streamlining of the system of response to these incidents (Section 8.4).
- A need to establish ongoing response funding to allow the above recommendation to be fulfilled and to provide for future incidents, which are not as likely to be understood as quickly as this one (Section 8.5).

9.1 Immediate actions (within weeks)

9.1.1 Public health

Erect signs to educate the public on the genuine issue and danger.

Education of the public of potential danger from sea life (with a picture of *P. maculata*).

9.1.2 Research and investigation

Conduct a survey of beach sediments and intertidal and shallow subtidal organisms within the Auckland region to confirm TTX is restricted to *P. maculata*.

Acknowledging that the high level of TTX within *P. maculata* in the affected area may be related to a food web effect (i.e. an increase in consumption of a particular food source that is, in turn, high in TTX), we recommend a wider survey of intertidal and shallow subtidal organisms within the area, including sampling of other benthic organisms (e.g. limpets, whelks, nematodes, flatworms), prey items (e.g. anemones), omnivorous grazers (such as kina), crustaceans (e.g. crabs and crayfish), and some species of fish that associate with the nearby rocky reefs (e.g. Banded wrasse, spotties). Previous studies have also identified the presence of TTX within beach sediments that can harbour toxic bacteria (Kogure et al.1988; Do et al. 1990), and it is recommended that further testing of sediments is done in the area. Preliminary investigations and results from analysis of shellfish in the area indicate that it is unlikely that TTX is within some commonly consumed seafood. However, a more detailed survey in the immediate area that includes a range of other organisms within the food web that may on occasion be consumed by people is warranted in order to fully alleviate concerns around human health risks.

Conduct a survey of *P. maculata* and TTX levels within the Auckland region to determine the extent to which the problem is localised.

Pleurobranchaea maculata is widely distributed in shallow subdital areas around New Zealand. As with many opisthobranchs, *P. maculata* can be difficult to find and is highly cryptic, but it can be observed laying long white tubular egg sacs during spring; often out in the open. A wider survey of *P. maculata* to determine its relative abundance and the spatial extent of high levels of TTX would enable a greater understanding of the extent of the problem; and whether the coincidence of increased dog deaths is related to the beaches in question being used more for dog walking than other areas, rather than an increase in the numbers of *P. maculata* on the beach.

Continue the aquarium based studies of *P. maculata* and TTX.

Pleurobranchaea maculata collected during this event were transported live to Nelson and some specimens are held in aquariums in Nelson. Some of these have recently spawned and if these are able to be raised their TTX levels may provide definitive proof about the origin of TTX in *P. maculata*.

Improve methodology to measure TTX, including completing mass balance studies.

It will be necessary to reduce the detection limit of the LC-MS test method to avoid false negative results. In addition we need to check that all the toxicity measured by mouse bioassay is accounted for by TTX to confirm no other toxin is present.

Continued monitoring for beach-cast *P. maculata*.

9.2 Medium-term actions (within months)

9.2.1 Public health

Develop an inter-agency management framework

In accordance with other central government initiated responses such as, marine toxin surveillance and recreational bathing guidelines.

Improve 0800 free phone systems.

Review the use of the MAF 0800 free phone for all unusual animal events and streamline inter agency communication. A similar review of the Public Health systems could also be undertaken to ensure relevant expertise is being drawn on quickly.

Emergency response funding.

Emergency response and contingency funding for similar events needs to be funded either regionally or nationally as it is unlikely that this quick resolution will be repeated. However it is likely that information gained from any investigation will have national significance.

9.2.2 Research and investigation

A nationwide assessment of TTX levels within *P. maculata* from various regions of the around New Zealand.

This study in concert with the subtidal survey make help establish variables that "trigger" high levels of TTX production, potentially enabling periods of "high risk" to be predicted. Investigate potential control measures, such as removal of egg masses from popular beaches.

9.3 Long-term actions (over a period of years)

9.3.1 Public health

Contingency funding for future events.

Continued surveillance of *P. maculata* and other organisms found to contain TTX as part of routine monitoring of beaches around New Zealand.

9.3.2 Research and investigation

Ecological studies in relation to *P. maculata* and TTX in concert with systematic sampling and analysis of benthic communities to attempt to identify the source of TTX and understand variables regulating its production.

Investigations into symbiotic bacteria within *P. maculata* and how these vary between highly toxic and less toxic specimens. Ultimately, these may lead to a better understanding of variables influencing levels of TTX in *P. maculata.*

Genetic studies of sodium channels in *P. maculata.* – i.e. why doesn't the toxin effect the sea slug?

9.4 A framework for future response

An important point to be raised from the current study is that the identification of a marine toxin within a week, enabling public awareness to be raised and potential human health risks to be minimised, is unlikely to be repeated. In most cases, the identification of the source of marine toxins affecting animal and human populations can take many months. For example, it took five months of testing in 1993 before a cluster of cat deaths was related to the consumption seafood laden with a toxic micro-alga. However, this event and the response of the local and central government agencies raises important issues for future situations where the time-frame to identify the toxin/toxins responsible may be considerably longer, or when direct impacts to humans may or may not eventuate. Clearly, a co-ordinated rapid local response is critical. In the current situation, because the MAF free phone number was seen as a first point of call for local veterinarians, local authorities were able to be alerted quickly and the public warned within a short period of time. If the toxin had not been identified quickly, however, extended testing would have been required.

9.5 Contingency funding for future response

Even though in this case the toxin has been identified quickly, still more work is required to eliminate concerns over other potential sources of TTX and to determine whether this toxin could make its way into the coastal food-chain; potentially posing a human-health risk. Consequently, for this and future toxin issues, which are of national significance, there is a need for contingency funding and systems to allow for, a more stream-lined response, longer-term testing (when toxins are harder to find), continued public education and continued warnings to keep people off beaches for longer; ensuring complacency does not prevail and to minimise human-health risks.

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12 Appendices

12.1 Appendix A: Agencies involved in the Steering group

Organisation	Group	Name	Phone	Mobile	Email
Auckland Regional Public Health Service	Communications	Sally Young	623 4600 x 27135	021 938 431	sallyy@adhb.govt.nz
		Manu Keug	6234600 xtn 26696	021 243 0615	manuk@adhb.govt.nz
	Steering Group	John Whitmore			johnwh@adhb.govt.nz
	TAG	Denise Barnfather		021 555 481	dbarnfather@adhb.govt.nz
MAF Biosecurity NZ	Communications	Annie Wright	04 894 0654	029 894 0654	annie.wright@maf.govt.nz
	Steering Group	Caleb King			caleb.king@maf.govt.nz
	TAG	Caleb King			
DOC	Communications	Amy Cameron	307 4846		acameron@doc.govt.nz
	Steering Group	Antonia Nichol	3074869	0272407827	anichol@doc.govt.nz
		Brett Butland			bbutland@doc.govt.nz
		Sean Goddard	3074824		sgoddard@doc.govt.nz
	TAG	Bill Trusewich			btrusewich@doc.govt.nz
Auckland Region Emergency Management Office	Communications	Kiri Maxwell	366 2000 x8700	027 225 5902	kiri.maxwell@arc.govt.nz
Auckland Regional Council	Communications	Janine Brennan-Lynch	366 2000 x8114	021 417 188	janine.brennan-lynch@arc.govt.nz

	Steering Group	Louise Mason			louise.mason@arc.govt
	TAG	Grant Barnes			grant.barnes@arc.govt.nz
		Megan Stewart			megan.stewart@arc.govt.nz
		Amanda Peart			amanda.peart@arc.govt.nz
	Steering Group Support	Cathy Klouwens			cathy.klouwens@arc.govt.nz
North Shore City Council	Communications	Kelly Gunn	09 486 8458	027 480 6149	kelly.gunn@northshorecity.govt.nz
	Steering Group	Duffy Visser			duffy.visser@northshorecity.govt.nz
	Steering Group	Ciaran Edwards			Ciaran.edwards@northshorecity.govt.nz
Auckland City Council	Communications	Angelika Doughty	373 6883	027 474 3695	angelika.doughty@aucklandcity.govt.nz
		John Evans	09 307 6080; Extn 7880	0274 545 637	john.evans@aucklandcity.govt.nz
	Steering Group	Darryl Thompson	3539667	0272890503	darryl.thompson@aucklandcity.govt.nz
		Chris Dee			Chris.dee@aucklandcity.govt.nz
	TAG	Richard Jacob-Hoff			richard.jakob-hoff@aucklandcity.govt.nz
Rodney District Council	Communications	Mike Isle	0800 426 5169	021 280 6445	mike.isle@rodney.govt.nz
Manukau City Council	Communications	Sharleen Pihema	262 5180	027 2188 285	sharleen.pihema@manukau.govt.nz
	Steering Group	James Corbett	262 8900 Extn 8135	Mob 0274 735 036	jcorbett@manukau.govt.nz
Ministry of Fisheries	Steering Group	John Taunton-Clark,	8207687	021 758434;	john.taunton-clark@fish.govt.nz
	Communications	Barbara Crocker	04 819 4218	021 388 659 l	barbara.crocker@fish.govt.nz

	TAG	Richard Ford	(04) 8194664		Richard.ford@fish.govt.nz.
Northland Regional Council	Steering Group	Jacquie Reed	09 470 1108	027 470 1182	jacquier@nrc.govt.nz
Cawthron Institute	Communications				
	Steering Group	Mark Jarvis			mark.jarvis@cawthron.org.nz
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Gribbles Pathology	TAG	Keith McSporran			Ketih.mcsporran@gribbles.co.nz
Environment Waikato	Steering & TAG TAG	Dominique Noiton	07 859 0721	021 241 6815	dominique.noiton@ew.govt.nz
		Bill Vant	859 0925		bill.vant@ew.govt.nz
	Communications	Karen Bennett	859 0922		Karen.bennett@ew.govt.nz
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	TAG	Hoe Chang			h.chang@niwa.co.nz
National Poisons Centre	TAG	Leo Schep			Leo.schep@stonebow.otago.ac.nz
NZ Food Safety Authority	Steering & TAG	Phil Busby	04 894 2635		Phil.busby@nzfsa.govt.nz
Waikato District Health Board	Steering & TAG	Dell Hood	07 8581063	021521 926	hoodd@waikatodhb.govt.nz

12.2 Appendix B: Details of the probable cases in dogs and their clinical signs (reproduced courtesy of Caleb King, MAF BNZ)

Case	Date	Place	Death	Vomiting	Ataxia	Lethargy	Salivation	Diarrhea	Muscle	Seizure	Resp	Cardiac
1	01/07/09	Cheltenham Beach	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes
2	07/07/09	Narrow Neck Beach	No	Yes	No	No	Yes	No	No	No	No	Yes
3	09/07/09	Narrow Neck Beach	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes
4	15/07/09	Eastern Beach	No	Yes	Yes	Yes	Yes	Yes	No	No	?	?
5	25/07/09	Waiake Beach	No	Yes	Yes	Yes	Yes	Yes	Yes	No	?	?
6	30/07/09	Long Beach	No	Yes	Yes	Yes	Yes	Yes	No	No	?	?
7	30/07/09	Browns Bay	No	Yes	Yes	No	No	Yes	No	No	?	?
8	01/08/09	Waiomu Beach,	No	Yes	Yes	Yes	Yes	Yes	No	No	?	Yes
9	02/08/09	Onetangi Bay	No	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Yes
10	03/08/09	Narrow Neck Beach	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes
11	03/08/09	Narrow Neck Beach	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes
12	7/08/09	Takapuna beach	No	Yes	Yes	Yes	Yes	No	Yes	No	No	Yes
13	22/08/09	Waiomu	No	Yes	Yes	Yes	Yes	No	Yes	No	No	No
14	06/08/09	Tamaki estuary	Yes	?	?	?	?	?	?	?	?	?

12.3 Appendix C: Situation reports 7 – 21 August 2009

BIOSECURITY NEW ZEALAND

Animals affected on Auckland beaches, 7 August 2009
National Centre for Biosecurity and Infectious Disease

Ref:

SITUATION REPORT

Investigation:	**Initiated**: 3 August 2009
Date of Report: 7 August 2009	**RC Controller:**
Date of Last Report:	**Number: 1**

Table of Contents

1 Situation Report - Overview

Current Objectives :

1.1 Protect the public
1.2 Delimit the extent of the cases
1.3 Determine origin - is there a biosecurity risk?
1.4 Look for other exotic organisms

- The incursion investigation team at the National Centre for Biosecurity and Infectious Disease, have commenced a surveillance and casing project for the dogs and penguin calls to the MAF 0800 80 99 66 free phone number that have been received since the public awareness was raised. To date 24 calls of veterinarians have been completed and 24 calls to people reporting dead penguins have been completed

- The National Poisons Centre in Dunedin, Otago have been requested to assist with the creation of a short list of toxins that we can test for and assist with the development of a sampling plan for the affected animals, potential vectors and environmental samples required to screen for a potential cause.

- The National Institute of Water and Atmospheric Research (NIWA) have been asked for assistance with the screening of satellite images of the Hauraki Gulf for evidence of water changes and conditions that may indicate a likelihood of a toxic algal bloom. This will be done using information about water temperatures and reflected light spectra that may indicate the presence or absence of chlorophyll. NIWA have contributed by identifying the jelly fish that were found on Narrow Neck beach near where the second dog was thought to become affected.

- The Cawthron Institute have tested samples from two of the dogs that have died, these samples are negative for blue green algae, further testing of potential vector species and environmental samples will continue next week.

- The New Zealand Centre for Conservation Medicine have been requested to provide post mortem and sampling assistance for any submissions of penguins that are found freshly dead in the coming two weeks.

- The New Zealand Veterinary Association has contacted veterinarians in the Auckland region and requested them to phone the MAF 0800 80 99 66 phone number to report any cases of dogs showing unusual clinical signs immediately after exposure to the beaches in the Auckland region. This action will enhance passive surveillance.

- Massey University, Albany has a lecturer in marine ecology, in the Coastal Marine Research Group who has received reports of dolphin mortality. The details of the number and location of these reports will be collected and if there are any fresh specimens for post mortem, specimens for histology will be sent to the pathologists at Massey University for histopathology, and other tests if required.

- North Shore City Council has erected signage on the North Shore beaches to inform people not to take their dogs on the beach.

- Laboratory results for dogs affected thus far are inconclusive, more testing is underway and results are expected in the next week. A coordinated sampling approach will be needed to rule out a range of natural and man-made compounds as causative agents.

2 Surveillance summary

- The area involved predominantly includes beaches on the Eastern coastline of the Auckland and Rodney districts for the species reported, dogs, penguins, pilchards. The data on dolphins is pending.
- There are 24 reports of sick dogs, of which at this stage 10 are considered cases
- There have been 24 reports of dead penguins on beaches
- Of the dog case reports 3 have died and 1 has been euthanased due to its deterioration.
- There are two clinical syndromes observed in dogs, one is a sudden onset of repeated vomiting at the beach followed by death within hours or rapid recovery, the other is the onset of neurological symptoms including seizures, unusual behaviour and altered mental status within hours of being at the beach and gradual recover or deterioration.
- A request to stakeholders that may have staff at beaches will be made to assist with the submission of penguins to the New Centre for Conservation Medicine for sampling. Animals submitted for post mortem need to be fresh, not decaying carcasses.

Appendices
Appendix 1 Maps of the report cases

3 Report from National Poisons Centre – Leo Schep

Intelligence Report

Over the last month isolated incidents of dog and marine life deaths have occurred on several beaches on the east coast of Auckland. Various causes have been considered including rat bait, exposure to jellyfish, such as Cyanea spp, and toxic algae of unknown origin.

Rat baits are typically second-generation coumarins that act by preventing blood clotting. Anticipated signs and symptoms would be evidence of bleeding such as haematemesis and melena. None of the intoxicated dogs demonstrated such symptoms.

The ingestion of jellyfish would lead to clinical effects such as dysphagia, stomach cramps, persistent vomiting and evidence of an allergic reaction. Again, animals that were poisoned did not present with these symptoms. Furthermore, given the relatively low toxicity of species found in New Zealand, compared with those in Australia, animals are anticipated to make a full recover. Finally, such exposures cannot explain the massive number of pilchards that have died or the increased number of mortalities in the dolphin populations.

Given the widespread geographical distribution of poisoning, the causative agent is most likely to be biological and possibly could be due to toxic algae. Various algae and

dinoflagellates can produce four major classes of marine toxins: saxitoxin, domoic acid, brevetoxin and okadaic acid. Of these, saxitoxin and brevetoxin can cause paralytic shellfish poisoning (PSP). These two should be the primary focus of the report (though very brief summaries could be considered for the others). Profiles for the PSPs will include the following sections, summarising information published in the peer-reviewed literature:

Define the toxins responsible
Identify the primary and secondary vectors (important to determine which species to assay for presence of the toxins)
Brief summary of the mechanism of action
Toxicokinetics
Signs and symptoms
  Onset and duration
  Routes of exposure (ingestion, skin, inhalation)
  Anticipated clinical effects and target organs
Methods of analysis - sample preparation and analysis (LCMS, receptor-binding assay)
Environmental fate – movement through and concentrations in the food chain (what are reported concentrations in the viscera of fish, for example, and what reported concentrations are necessary for the poisoning of species, e.g. sea birds, dolphins and other mammals such as dogs)

Appendices
Appendix 2 Algal bloom saxotoxin

4 *Report from the National Institute of Water and Atmospheric Research*

Jellyfish identification

The jellyfish found on Narrow Neck beach is a new species of Cyanea, currently being described by Dennis Gordon and Lisa Gershwin. It's a native species and occurs around central New Zealand.

NASA Ocean colour imagery of Hauraki Gulf, July – August 2009

Precis: Chlorophyll absorbs blue light strongly, but not green light. The ratio of blue to green reflectance therefore provides an indicator for chlorophyll biomass. Quantitative values are derived using an empirical algorithm developed using thousands of data points from open ocean waters around the globe. This algorithm is compromised if substances other than chlorophyll are present and absorbing or scattering strongly in the blue-green – this includes dissolved organic matter, e.g. land run-off, and suspended sediments.

In the central Hauraki Gulf, the water generally appears to be dominated by chlorophyll (M.H. Pinkerton) but near-shore, the chlorophyll satellite product is almost certainly in error. Near-shore phytoplankton blooms may be distinguished using a time record of several weeks, during which chlorophyll levels increase steadily and then decline. Any chlorophyll signal which appears over a shorter time-scale, such as days, is likely to be caused by sediments washing in from rivers or resuspended by wind mixing. The data provided here were measured, processed and plotted by the Ocean Color Biology Group at NASA and provided to me as a free,

public domain data service. Please cite NASA when using the data.

Devonport: There's not much clear water between Devonport and the islands, the area is largely masked as being contaminated by reflectance from the land; Infer chlorophyll distributions from the clearer waters off-shore.

Evidence for a toxic bloom: We expect a toxic bloom to originate with the intrusion of warm water from off-shore (H. Chang): there is no evidence for such an intrusion in the sea surface temperature imagery (attached). There is evidence of bloom-level chlorophyll concentrations in the inner Hauraki Gulf, especially in the first 2 weeks of July (~4th to 17th). Later in July, distributions are much patchier in time and it is not clear whether sediments or chlorophyll is causing the elevated 'chlorophyll' product signal.

CAUTION: This is version 1 of this document. Hoe Chang and Matt Pinkerton have yet to add their comments/corrections to my interpretation. This report should not be reported wider until an expert opinion has been completed and the interpretation confirmed.

Appendices – Satelite imagery from NASA

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5 Report from the Cawthron Institute

Samples received to date:

1. Dried seaweed – Narrowneck beach – from Ciaran Edwards (NSCC)
– sample screened for potentially toxic algae/cyanobacteria – none found
- sample extracted to be send for mouse bioassay (probably results towards end of next week)

2. Damp seaweed – many types from Narrowneck beach collected by Bill Trusewich (DOC)
– samples screened for potentially toxic algae/cyanobacteria – none found
– samples washed and water screened for potentially toxic algae/cyanobacteria – none found
- sample extracted to be send for mouse bioassay (probably results towards end of next week)

3. Dog vomit samples from Keith McSporran Gribbles Veterinary – 3 samples
– samples screened for potentially toxic algae/cyanobacteria – none found
- selected 2 samples and have been extracted to be send for mouse bioassay (probably results towards end of next week)

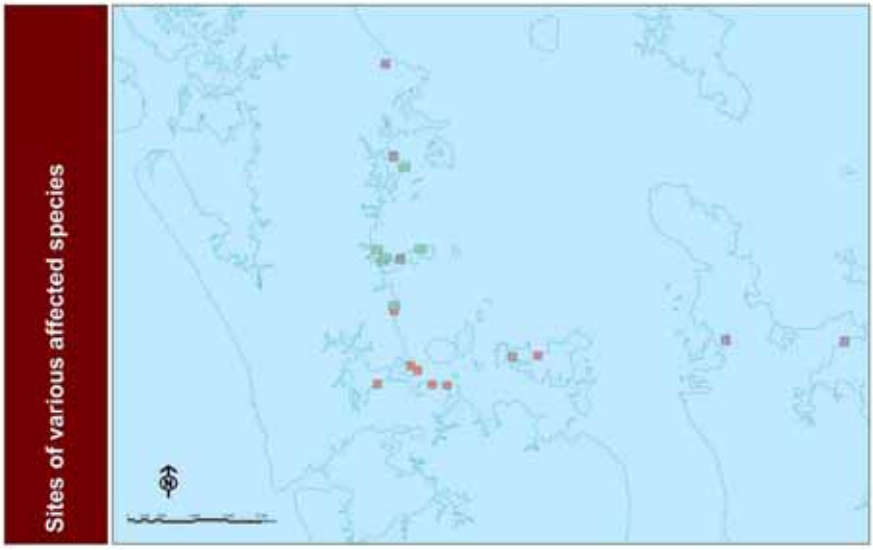
That's all so far

Also samples arriving from ARC for phytoplankton identification today

Hoping to get a jellyfish today as well?

6 Appendix 1 Maps of report cases


Whangaparaoa Bay
Orewa
Long Bay
0
1,125
2,250
4,500
Meters


Sites of various affected species

7 Appendix 2 Algal bloom saxotoxin

Algal Bloom - Saxitoxin

Saxitoxin is produced by certain species of dinoflagellates, including *Alexandrium catenella*, *A. tamarense*, *Pyrodinium bahamense*, and *Gymnodinium catenatum*. Filter-feeding shellfish consume these and, while not being affected themselves, may intoxicate animals and humans that eat these shellfish. Saxitoxin has also, occasionally, been isolated in pufferfish.

While high levels of saxitoxin has been associated with "algae bloom" or "toxic red tide", elevated levels of algae can occur without the characteristic sea color changes.

Poisoning will usually only be seen following the ingestion of seafood that is harvested during periods of high algae concentrations. This is most likely to occur during the spring or fall (autumn), but may also occur during summer months. Poisoning can occur whether the shellfish are cooked or raw.

Clinical effects

Effects are initially seen as paresthesia in and around the mouth. This spreads to the face and neck, and may be followed by weakness and paralysis of limbs. Nausea, vomiting, and diarrhea are reported, and a sensation of floating is frequently experienced. Death may occur due to respiratory paralysis, the most concerning aspect of this poisoning. Central nervous system depression is not characteristic of saxitoxin exposure.

A brief summary of anticipated symptoms are provided in table 1.

Onset of symptoms from saxitoxin ingestion generally occurs in about 20 to 60 minutes though they may not become evident for up to 3 hours. The earlier the onset of symptoms the more serious the effects are likely to be.[5] Death may occur in 2 to 12 hours in severe cases not receiving medical care.

Table 1 Common anticipated symptoms following the ingestion of saxitoxin

Mild Saxitoxin Toxicity	Moderate Saxitoxin Toxicity	Severe Saxitoxin Toxicity
Perioral/lingual numbness and tingling	Paresthesia of the face and neck	Weakness of arms and legs
	Nausea and vomiting	Respiratory depression
	Diarrhea	Dysphagia
	Headache	Dysarthria
	Ataxia	Limb paralysis
	Sensation of floating	Respiratory paralysis

Furthermore, the cardiac effects which may occur include reduction in cardiac output, tachycardia, bradycardia, hypotension, hypertension, ECG changes.

Toxicity
Toxicity of the two salts:

Saxitoxin dihydrochloride

LD50 Oral, Mouse 0.263 mg/kg
LD50 IV, Mouse 0.0034 mg/kg
LD50 IP, Mouse 0.010 mg/kg

Saxitoxin hydrate

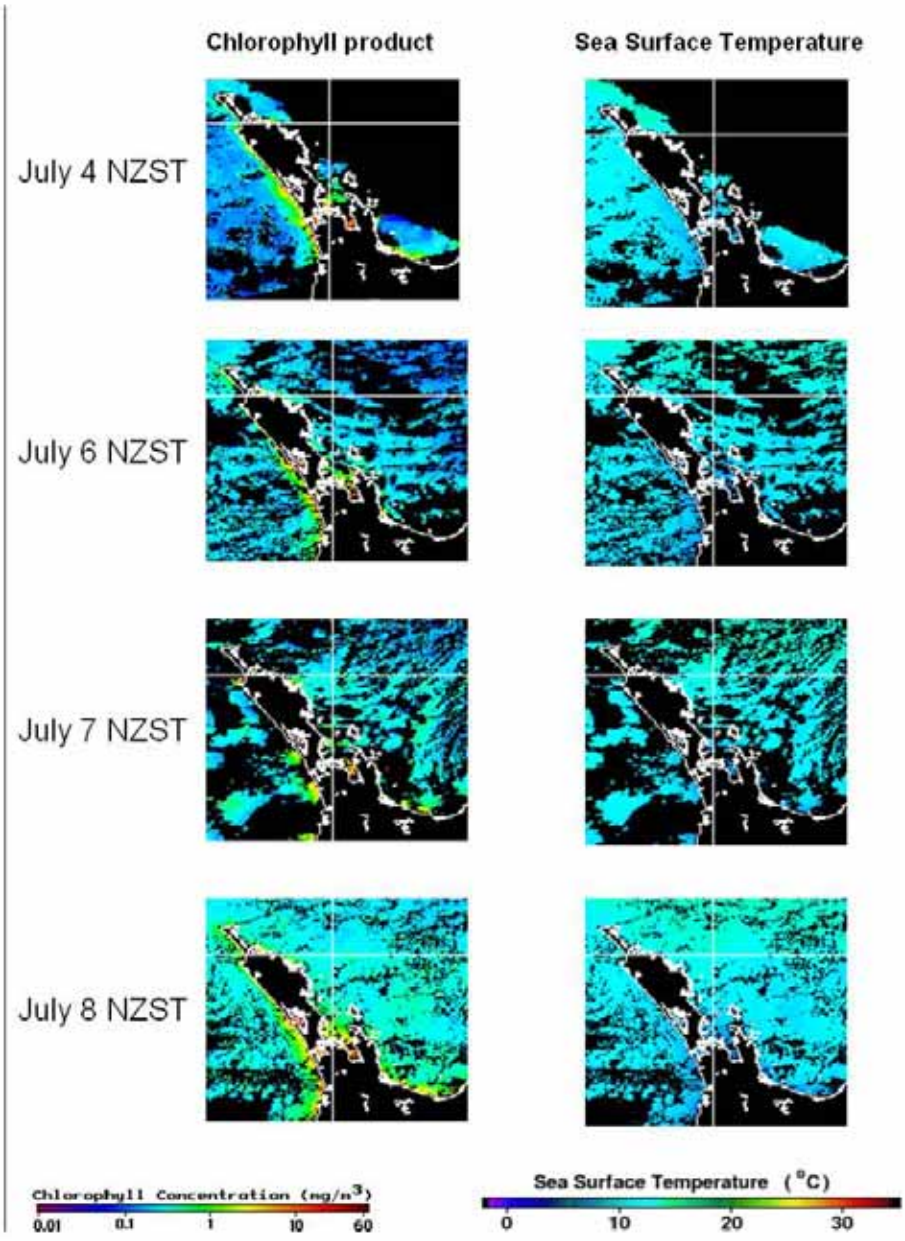
LD50 SC, Mouse 0.0165 mg/kg
LD50 IV, Mouse 0.008 mg/kg
LD50 IP, Mouse 0.005 mg/kg

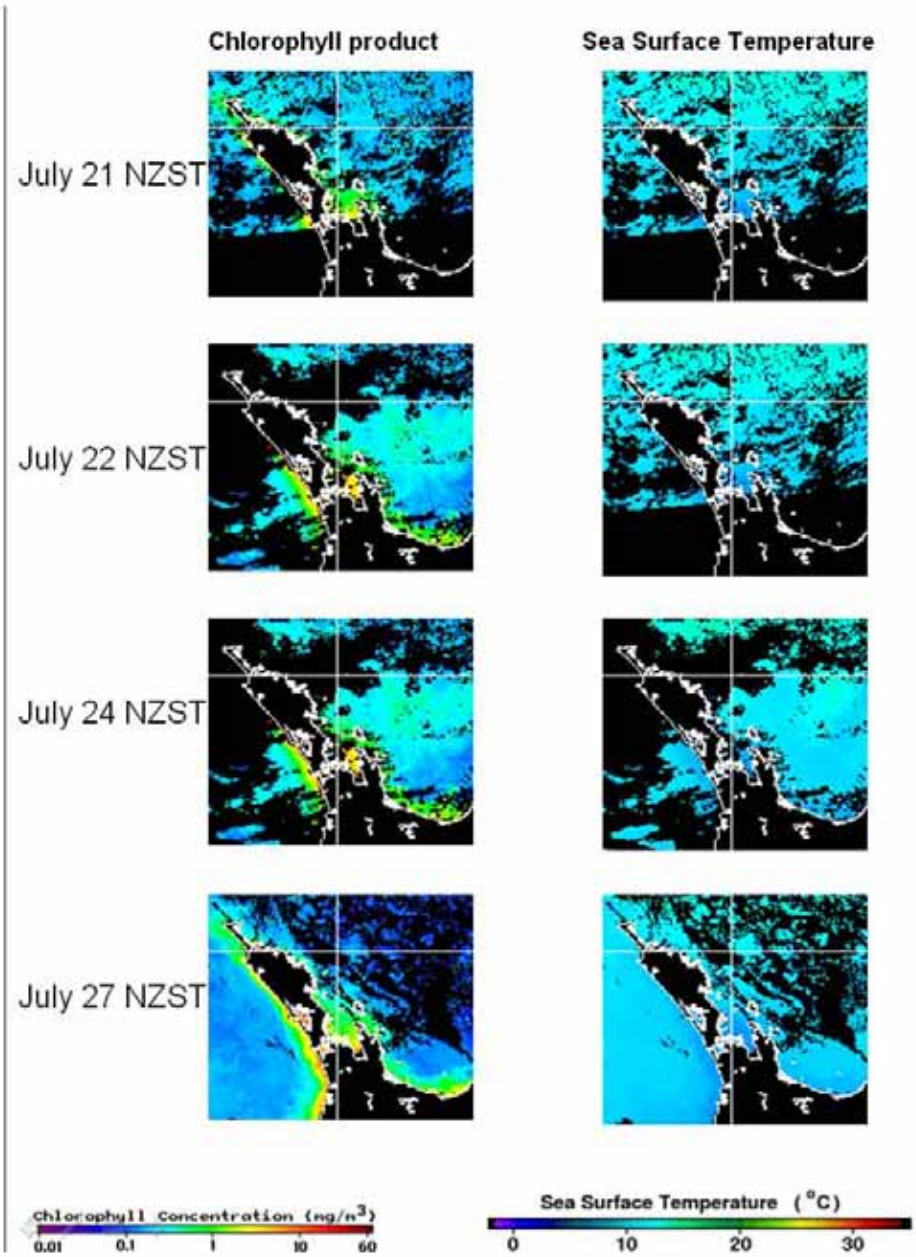
Toxic mechanism of action

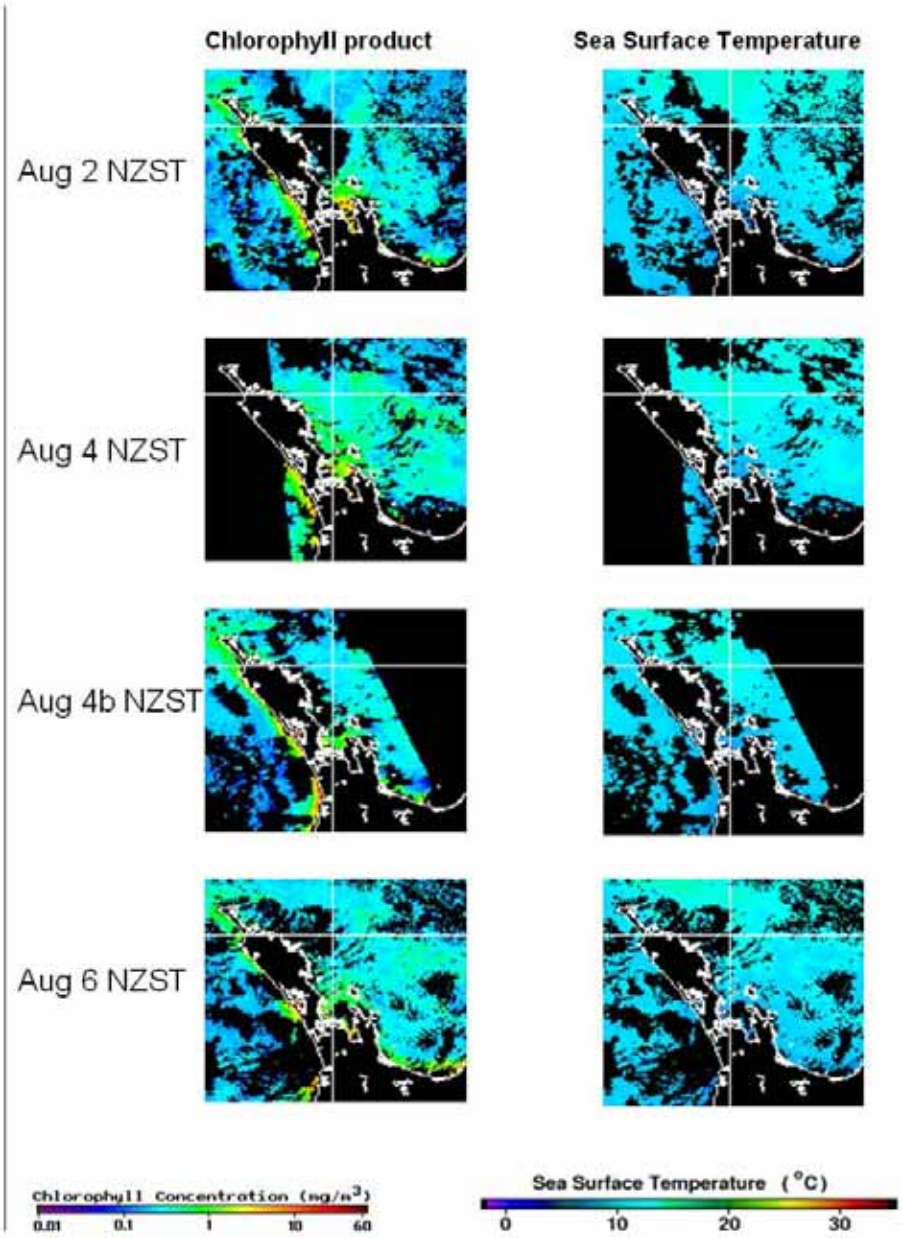
Saxitoxin is a sodium-channel inhibitor in excitable membranes of the nervous system. This blocks the transmission of nerve impulses at the axonal head and muscular membrane of neuromuscular junctions. The effects of this are prolonged distal latencies, reduced conduction velocities, and a reduction of motor and sensory amplitudes in peripheral nerves. In particular AV nodal conduction is suppressed, and the medullary respiratory center is depressed.

There is little effect on neuronal junctions of the central nervous system

8 Appendix 3 NASA Ocean Imagery




Chlorophyll product
Sea Surface Temperature
July 21 NZST
July 22 NZST
July 24 NZST
July 27 NZST
Chlorophyll Concentration (mg/m3)
0.01
0.1
1
10
60
Sea Surface Temperature (°C)
0
10
20
30


Chlorophyll product
Sea Surface Temperature
Aug 2 NZST
Aug 4 NZST
Aug 4b NZST
Aug 6 NZST
Chlorophyll Concentration (mg/m3)
0.01
0.1
1
10
60
Sea Surface Temperature (°C)
0
10
20
30

Appendix 4 Serum biochemistry from dogs

@HEADER
ACCESSION AU0916797
REPORT BIOCH
REPORTSTATUS FINAL
OWNER OBORN,RACHELLE
SUBREF B251865
SPECIES Canine
BREED Border Collie
SEX Female
AGE 10 MONTH(S)
SENT 3/08/2009 11:58:39 AM
RECEIVED 4/08/2009 11:58:39 AM
SIGNEDDATE 4/08/2009 1:06:13 PM
SUBMITTER Caleb King
TECHNICIAN CROBER
LABORATORY Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1 37-41 Carbine Road
LABADDR2 Mt Wellington
REPORTFEE
@RESULTS

ROXY 13599	NA	146	MMOL/L		(145 - 160)
ROXY 13599	K	4.9	MMOL/L		(3.7 - 5.7)
ROXY 13599	NAKR	29.8	RATIO		
ROXY 13599	CL	112	MMOL/L		(98 - 122)
ROXY 13599	CRE	63	UMOL/L		(25 - 102)
ROXY 13599	URE	6.7	MMOL/L		(2.3 - 8.7)
ROXY 13599	PO4	2.21	MMOL/L		(0.8 - 2.8)
ROXY 13599	CHOL	6.2	MMOL/L		(2.7 - 9.2)
ROXY 13599	TP	60	G/L		(47 - 71)
ROXY 13599	ALB	38	G/L		(28 - 41)
ROXY 13599	GLO	22	G/L		(15 - 34)
ROXY 13599	AGR	1.73	RATIO		(0.8 - 2.1)
ROXY 13599	CA	1.95	MMOL/L	L	(2.16 - 2.98)
ROXY 13599	BILI	1.3	UMOL/L		(0 - 4.2)
ROXY 13599	ALP	70	IU/L		(10 - 160)
ROXY 13599	ALT	70	IU/L		(15 - 110)
ROXY 13599	AST	86	IU/L	H	(15 - 55)
ROXY 13599	CK	273	IU/L		(0 - 506)
ROXY 13599	AMY	793	IU/L		(250 - 1060)

@COMMENTS
BIOCHEMISTRY-HITACHI performed at Gribbles Veterinary Pathology Ltd-Auckland Reference Ranges and Method Reference will be supplied on request
Testing Requested
1 x Sick Canine Panel
1 x Sick Canine Panel
@END

```
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ACCESSION    AU0916782
REPORT       HAEM
REPORTSTATUS       FINAL
OWNER SINGH,BILL
SUBREF
SPECIES      Canine
BREED Blue Heeler
SEX    Male castrate
AGE    18 MONTH(S)
SENT   2/08/2009 8:50:06 AM
RECEIVED     4/08/2009 8:50:06 AM
SIGNEDDATE   4/08/2009 11:46:38 AM
SUBMITTER    Caleb King
TECHNICIAN   JMEYER
LABORATORY   Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1     37-41 Carbine Road
LABADDR2     Mt Wellington
REPORTFEE
@RESULTS
MAX    RBC    6.69   X 10^12/L          (5.5 - 8.2)
MAX    HB     148    G/L          (120 - 180)
MAX    HCT    0.47   L/L          (0.37 - 0.55)
MAX    MCV    70     FL           (60 - 78)
MAX    MCH    22     PG           (20 - 25)
MAX    MCHC   314    G/L          (310 - 360)
MAX    NRC    1      /100LEU      H      <0
MAX    WBC    15.8   X 10^9/L     H      (6 - 15)
MAX    NEUT   84     %
MAX    NEUTAB       13.3   X 10^9/L     H      (3.6 - 11.5)
MAX    LYMPH  8      %
MAX    LYMPHAB      1.3    X 10^9/L           (1 - 4.8)
MAX    MONO   8      %
MAX    MONOAB       1.3    X 10^9/L           (0.2 - 1.5)
MAX    WBCRAW       16.0   X 10^9/L           (6 - 17)
@COMMENTS
EDTA sample is 1 day old, this can result in changes to WBC morphology and
selected red cell parameters.
Poikilocytes 2+ with echinocytes.
Platelets appear normal in number
Platelets are clumped in the film
No fresh film received.

krm

VETERINARY INTERPRETATION:

Marginal increase in mature neutrophils may be due to stress or possibly
low-grade inflammation. The significance of the 2+ poikilocytes
(ecchinocytes - rbcs with spicules) is unclear. Likely an artefact during
smear preparation of an aged sample.

Jon Meyer
BVSc, DVSc, Diplomate ACVP
HAEMATOLOGY - GENERAL performed at Gribbles Veterinary Pathology Ltd-
Auckland Reference Ranges and Method Reference will be supplied on request
Testing Requested
1 x Complete Blood Count
1 x Complete Blood Count
@END
```

@HEADER
ACCESSION AU0916782
REPORT BIOCH
REPORTSTATUS FINAL
OWNER SINGH,BILL
SUBREF
SPECIES Canine
BREED Blue Heeler
SEX Male castrate
AGE 18 MONTH(S)
SENT 2/08/2009 8:50:06 AM
RECEIVED 4/08/2009 8:50:06 AM
SIGNEDDATE 4/08/2009 10:26:10 AM
SUBMITTER Caleb King
TECHNICIAN JMEYER
LABORATORY Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1 37-41 Carbine Road
LABADDR2 Mt Wellington
REPORTFEE
@RESULTS

MAX	NA	151	MMOL/L		(145 - 160)
MAX	K	4.2	MMOL/L		(3.7 - 5.7)
MAX	NAKR	36.0	RATIO		
MAX	CL	115	MMOL/L		(98 - 122)
MAX	CRE	70	UMOL/L		(25 - 102)
MAX	URE	8.7	MMOL/L		(2.3 - 8.7)
MAX	PO4	2.44	MMOL/L		(0.8 - 2.8)
MAX	CHOL	6.2	MMOL/L		(2.7 - 9.2)
MAX	TP	64	G/L	(47 - 71)	
MAX	ALB	38	G/L	(28 - 41)	
MAX	GLO	26	G/L	(15 - 34)	
MAX	AGR	1.46	RATIO		(0.8 - 2.1)
MAX	CA	2.41	MMOL/L		(2.16 - 2.98)
MAX	BILI	1.5	UMOL/L		(0 - 4.2)
MAX	ALP	41	IU/L	(10 - 160)	
MAX	ALT	46	IU/L	(15 - 110)	
MAX	AST	84	IU/L H	(15 - 55)	
MAX	CK	410	IU/L	(0 - 506)	
MAX	AMY	653	IU/L	(250 - 1060)	

@COMMENTS
VETERINARY INTERPRETATION:

Mild/non-specific changes on the biochemistry. CBC still to follow....

Jon Meyer
BVSc, DVSc, Diplomate ACVP
BIOCHEMISTRY-HITACHI performed at Gribbles Veterinary Pathology Ltd-
Auckland Reference Ranges and Method Reference will be supplied on request
Testing Requested
1 x Sick Canine Panel
1 x Sick Canine Panel
@END

```
@HEADER
ACCESSION    AU0914878
REPORT       ADMIN
REPORTSTATUS       FINAL
OWNER LEIGH,JENNIFER
SUBREF       INV 2211
SPECIES      Canine
BREED Beagle
SEX    Male castrate
AGE    2 YEAR(S)
SENT   10/07/2009 3:18:00 PM
RECEIVED     10/07/2009 3:18:00 PM
SIGNEDDATE   22/07/2009 3:58:15 PM
SUBMITTER    Caleb King
TECHNICIAN   KCOOPE
LABORATORY   Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1     37-41 Carbine Road
LABADDR2     Mt Wellington
REPORTFEE
@RESULTS
GEORGE 12610       MISC  See below
@COMMENTS
GEORGE DOG BEAGLE VOMIT

MICROALGAE ANALYSES

MICROALGAE = ABSENT
(Method = Presence/Absence: Unesco 1978 Modified)

Results apply to samples as received.  Our routine detection limits for
chemical testing relate to samples with a clean matrix.  REproted detection
limits may be higher for individual samples if there is insufficient sample
or the matrix is complex.

(Testing performed by Cawthron)

MISCELLANEOUS TESTS performed at Gribbles Veterinary Pathology Ltd-
Auckland Reference Ranges and Method Reference will be supplied on request
Testing Requested
1 x Miscellaneous Test Request
1 x Miscellaneous Test Request
@END
```

```
@HEADER
ACCESSION    AU0914878
REPORT       ADMIN
REPORTSTATUS       INTERIM
OWNER LEIGH,JENNIFER
SUBREF       INV 2211
SPECIES      Canine
BREED Beagle
SEX    Male castrate
AGE    2 YEAR(S)
SENT   10/07/2009 3:18:00 PM
RECEIVED     10/07/2009 3:18:00 PM
SIGNEDDATE
SUBMITTER    Caleb King
TECHNICIAN
LABORATORY   Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1     37-41 Carbine Road
LABADDR2     Mt Wellington
REPORTFEE
@RESULTS
GEORGE 12610       MISC  See below
@COMMENTS
GEORGE DOG BEAGLE VOMIT

MICROALGAE ANALYSES

MICROALGAE = ABSENT
(Method = Presence/Absence: Unesco 1978 Modified)

Results apply to samples as received.  Our routine detection limits for
chemical testing relate to samples with a clean matrix.  REproted detection
limits may be higher for individual samples if there is insufficient sample
or the matrix is complex.

(Testing performed by Cawthron)

MISCELLANEOUS TESTS performed at Gribbles Veterinary Pathology Ltd-
Auckland Reference Ranges and Method Reference will be supplied on request
Testing Requested
1 x Miscellaneous Test Request
1 x Miscellaneous Test Request
@END
```

Appendix 5 PM and histology of Black backed gulls

@HEADER
ACCESSION AU0915008
REPORT HISTO
REPORTSTATUS FINAL
OWNER OMAHA BEACH
SUBREF IDC 2212
SPECIES Avian
BREED Black Backed Gull
SEX Unknown
AGE 0 UNKNOWN
SENT 13/07/2009 9:49:55 AM
RECEIVED 13/07/2009 9:49:55 AM
SIGNEDDATE 20/07/2009 12:16:31 PM
SUBMITTER Caleb King
TECHNICIAN CHARVE
LABORATORY Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1 37-41 Carbine Road
LABADDR2 Mt Wellington
REPORTFEE
@RESULTS
@COMMENTS
AU 09- 15008

Patient: Black Back Gulls x 3

Histopathology:
There is mild autolysis in all tissues.
Black Back Gull #2:
Lungs - There is moderate congestion.

Liver and Kidney - There are multiple chronic granulomas (circumscribed infiltrates of multinucleate and epithelioid macrophages with small numbers of lymphocytes and plasma cells) with intralesional refractile parasite remnants.

Intestines - There are small numbers of mucosal nematode parasites.

Lungs, Brain, Heart, Skeletal muscle, Proventriculus and Gizzard - no significant findings

Black Back Gull #1 Kidney - no significant findings

Black Back Gull #3 Kidney - no significant findings

Morphologic Dx:
Black Back Gull #1
Skin of lower legs and the feet - blue-green discolouration Urates - blue-green discolouration Contents of the Proventriculus and Gizzard - blue foreign material Lungs - Congestion and oedema, moderate, acute

Black Back Gull #2
Skin of lower legs and the feet - blue-green discolouration Contents of the Proventriculus and Gizzard - blue foreign material Lungs - Congestion, moderate, acute Liver and Kidney - Multiple chronic granulomas with intralesional parasites Intestines - Mucosal nematode parasites, mild

Black Back Gull #3

Dislocation of the head and vertebral column with haemorrhage, severe, acute Skin of lower legs and the feet - blue-green discolouration

Comment:
The multiple peracute deaths with foreign material and bread in the proventriculus and gizzard are suggestive of a toxicity/ poisoning. Cannot rule out metabolic toxin with no changes on histopathology.
There was no evidence of hemorrhage, gout or mineralization in the kidneys. I cannot rule out peracute renal tubular damage in the kidneys, as this is often does not have histopathology changes that correlate with clinical disease, and as the birds were dead for 48 hours before necropsy autolysis makes subtle changes difficult to discern.

Catherine Harvey B.V.Sc, Diplomate ACVP

HISTOLOGY performed at Gribbles Veterinary Pathology Ltd- Auckland
Reference Ranges and Method Reference will be supplied on request Testing Requested
1 x Histology Multiple/Necropsy tissues
1 x Histology Multiple/Necropsy tissues
@END

@HEADER
ACCESSION AU0915008
REPORT HISTO
REPORTSTATUS FINAL
OWNER OMAHA BEACH
SUBREF IDC 2212
SPECIES Avian
BREED Black Backed Gull
SEX Unknown
AGE 0 UNKNOWN
SENT 13/07/2009 9:49:55 AM
RECEIVED 13/07/2009 9:49:55 AM
SIGNEDDATE 20/07/2009 12:16:31 PM
SUBMITTER Caleb King
TECHNICIAN CHARVE
LABORATORY Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1 37-41 Carbine Road
LABADDR2 Mt Wellington
REPORTFEE
@RESULTS
@COMMENTS
AU 09- 15008

Patient: Black Back Gulls x 3

Histopathology:
There is mild autolysis in all tissues.
Black Back Gull #2:
Lungs - There is moderate congestion.

Liver and Kidney - There are multiple chronic granulomas (circumscribed infiltrates of multinucleate and epithelioid macrophages with small numbers of lymphocytes and plasma cells) with intralesional refractile parasite remnants.

Intestines - There are small numbers of mucosal nematode parasites.

Lungs, Brain, Heart, Skeletal muscle, Proventriculus and Gizzard - no significant findings

Black Back Gull #1 Kidney - no significant findings

Black Back Gull #3 Kidney - no significant findings

Morphologic Dx:
Black Back Gull #1
Skin of lower legs and the feet - blue-green discolouration Urates - blue-green discolouration Contents of the Proventriculus and Gizzard - blue foreign material Lungs - Congestion and oedema, moderate, acute

Black Back Gull #2
Skin of lower legs and the feet - blue-green discolouration Contents of the Proventriculus and Gizzard - blue foreign material Lungs - Congestion, moderate, acute Liver and Kidney - Multiple chronic granulomas with intralesional parasites Intestines - Mucosal nematode parasites, mild

Black Back Gull #3
Dislocation of the head and vertebral column with haemorrhage, severe, acute Skin of lower legs and the feet - blue-green discolouration

Comment:

The multiple peracute deaths with foreign material and bread in the proventriculus and gizzard are suggestive of a toxicity/ poisoning. Cannot rule out metabolic toxin with no changes on histopathology.
There was no evidence of hemorrhage, gout or mineralization in the kidneys. I cannot rule out peracute renal tubular damage in the kidneys, as this is often does not have histopathology changes that correlate with clinical disease, and as the birds were dead for 48 hours before necropsy autolysis makes subtle changes difficult to discern.

Catherine Harvey B.V.Sc, Diplomate ACVP

HISTOLOGY performed at Gribbles Veterinary Pathology Ltd- Auckland
Reference Ranges and Method Reference will be supplied on request Testing Requested
1 x Histology Multiple/Necropsy tissues
1 x Histology Multiple/Necropsy tissues
@END

@HEADER
ACCESSION AU0915008
REPORT NECRO
REPORTSTATUS FINAL
OWNER OMAHA BEACH
SUBREF IDC 2212
SPECIES Avian
BREED Black Backed Gull
SEX Unknown
AGE 0 UNKNOWN
SENT 13/07/2009 9:49:55 AM
RECEIVED 13/07/2009 9:49:55 AM
SIGNEDDATE 13/07/2009 3:22:47 PM
SUBMITTER Caleb King
TECHNICIAN CHARVE
LABORATORY Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1 37-41 Carbine Road
LABADDR2 Mt Wellington
REPORTFEE
@RESULTS
BLACK BACKED GULLS NEC nr -
@COMMENTS

AU 09- 15008

Patient: Black Back Gulls x 3
Black Back Gull #1 - Found dead at time of pick-up.
Black Back Gull #2 - Found dead at time of pick-up.
Black Back Gull #3 - Alive at time of pick-up (euthanised by breaking neck?)

Gross examination:
Black Back Gull #1
The 1.1kg male Black Backed Gull is in good post mortem and good body condition. The lower legs and the feet are discoloured light blue -green. There are light blue -green urates over the feather surrounding the vent. The proventriculus and gizzard contain a small amount of luminal light blue viscous material. The lungs are dark red and ooze serosanguineous fluid when cut, but float in formalin.

Black Back Gull #2
The 800 gm female Black Backed Gull is in good post mortem and good body condition. The lower legs and the feet are discoloured light blue -green. The proventriculus and gizzard contain large amount of white bread and small amounts of bright blue granular material. The lungs are dark red and ooze serosanguineous fluid when cut, but float in formalin.

Black Back Gull #3
The 1 kg female Black Backed Gull is in good post mortem and good body condition. The lower legs and the feet are discoloured light blue -green. The proventriculus and gizzard contain scant fragments of plant material. The lungs are pink and float in formalin. There is a 5 x 2 x 1 cm area of haemorrhage in the soft tissues of the cranial neck. There is a complete dislocation between the head and the vertebral column.

Morphologic Dx:
Black Back Gull #1
Skin of lower legs and the feet - blue-green discolouration Urates - blue-green discolouration Contents of the Proventriculus and Gizzard - blue foreign material Lungs - Congestion and oedema, moderate, acute

Black Back Gull #2
Skin of lower legs and the feet - blue-green discolouration Contents of the Proventriculus and Gizzard - blue foreign material Lungs - Congestion and oedema, moderate, acute

Black Back Gull #3
Dislocation of the head and vertebral column with haemorrhage, severe, acute Skin of lower legs and the feet - blue-green discolouration

Comment:
In gulls #1 and #2 there was no evidence of trauma or underling disease.
The blue foreign material in the proventriculus and gizzard and the discoloration of the skin of the lower legs and feet is suggestive of toxicity/ poisoning.
As per information from Caleb King MAF Biosecurity this may be DRC 1339 (3-chloro-p-toluidinehydrochloride) poisoning.

Digital images were taken of the legs of all three gulls, and of the stomach contents of gull #2.
Samples of liver, kidney, and the contents of proventriculus and gizzard, from each gull have been frozen. Please let the lab know if you wish to go ahead with any further testing, or what you would like done with the frozen tissues.
Samples of all tissue from all gulls have been fixed in formalin.
Histopathology is pending.

Catherine Harvey B.V.Sc, Diplomate ACVP

NECROPSY performed at Gribbles Veterinary Pathology Ltd- Auckland Reference Ranges and Method Reference will be supplied on request Testing Requested
1 x Necropsy
1 x Necropsy
1 x Necropsy
@END

List of Contacts

TBC

BIOSECURITY NEW ZEALAND

Animals affected on Auckland beaches, 13 August 2009
National Centre for Biosecurity and Infectious Disease

Ref:

SITUATION REPORT

Investigation: 2211 Dead dogs Auckland	**Initiated**: 3 August 2009
Date of Report: 13 August 2009	**ARC contact:** Grant Barnes
Date of Last Report: 7 August 2009	**Number: 2**

Table of Contents

1 *Situation Report - Overview*

Current Objectives :

1.1 Protect the public
1.2 Delimit the extent of the cases
1.3 Determine origin - is there a biosecurity risk?
1.4 Look for other exotic organisms

- The Auckland Regional Council are the lead agency for this response from the 10th August 2009 and are coordinating the technical, communications and management teams in an effective manner. Conference calls are on an as-required basis within each team and participants are kept informed at the major conference call twice each week.

- The incursion investigation team at the MAF National Centre for Biosecurity and Infectious Disease, have continued the surveillance and casing project for the dogs and penguin calls to the MAF 0800 80 99 66 free phone number that have been received since the public awareness was raised. A total of 210 calls have been received to date. There has been one report of illness in humans following the consumption of Tuatua, this case has been passed to the Northland District Public Health unit.

- The National Poisons Centre in Dunedin, Otago are providing expert advice on the toxicology, a report on the possible toxins that are causing clinical signs has been submitted.

- The National Institute of Water and Atmospheric Research (NIWA) have screened the satellite images in the last three months of the Hauraki Gulf for evidence of water changes and conditions that may indicate a likelihood of a toxic algal bloom. There have been no unusual, major blooms detected in the Hauraki Gulf in the last three months

- The Cawthron Institute have identified a high concentration of toxin in the grey-gilled sea slug – *Pleurobanchaea maculata* using the mouse bioassay test. This toxin has been provisionally identified as tetrodotoxin, a potent neurotoxin. Further testing is required to confirm this provisional result. The compound resembling tetrodotoxin has also been found in the vomitus of one dog. Testing will be carried out on specimens from other affected dogs to see if they have the same toxin. This is an unusual finding, and possibly a first report in New Zealand.

- The New Zealand Centre for Conservation Medicine has post mortemed five penguins to date. The gross post mortem results indicate that these birds are in poor body condition and starvation is the likely cause of death.

- Veterinarians in the Auckland region are continuing to phone the MAF 0800 80 99 66 phone number to report cases in dogs. There has been one report of case of a dog in the Thames region so the message to report dogs sick after being at the beach will be sent to a veterinarians in the Rodney and Waikato districts.

- Massey University, Albany has a lecturer in marine ecology, in the Coastal Marine Research Group who has received eight reports of dolphin mortality in the Hauraki Gulf during the period July –August 2009. The details of the number and location of these reports has been collected and specimens sent for histopathology to the pathologists at Massey University. Gut specimens from dolphins are available for testing should they be required.

- North Shore City Council has erected signage on the North Shore beaches to inform people not to take their dogs on the beach. Auckland City Council has erected signage on Eastern Suburb beaches

- ARC marine ecologists note that isolated incidences of pilchard and penguins deaths at this time of year are not ordinarily unusual and natural causes of death are a likely scenario.

2 Report from Auckland Regional Council

- Toxicity testing of dog tissue, dolphin, and pilchards gut contents. Testing has been commissioned with results anticipated later next week subject to delivery of samples from sources.

- Collection of pleurobranch specimens from Auckland beaches . Completed. See field report in appendix 1. High abundances recorded at Narrow Neck beach, only one individual found elsewhere. Most individuals collected were dead and over a wide size range.

- Collection of pleurobranch specimens from Waikato sites by NIWA . Undertaken today. Results not currently known.

- Speciation of toxin(s) from dog vomit and opisthobranch samples that triggered the positive bioassay result :Ongoing. One toxin identified as tetrodotoxin. The sea slug had very high concentrations of this toxin.

- Advice received from Dr Richard Wilan, curator of molluscs at the Museum and Art Gallery of the Northern Territory and an expert in Opisthobranchs.

Dr Wilan was surprised that it was *P. maculata*, which is not toxic in its own right and has not been associated with this level of toxicity or deaths before. They have washed up in numbers on several occasions around Auckland previously. They are annual and die after reproduction.

The toxin may be as a result of them ingesting and accumulating the toxin as a result of their scavenging behaviour. They are an opportunistic scavenger but not a grazer. They are unlikely to have feed directly on any plant material, rather they are more likely to ingest it inadvertently while consuming prey or something that has died. They will consume dead and dying material.

The size range of specimens collected yesterday was 25-80mm with the bulk being 30-50mm. This smaller size suggests the toxin is potentially killing *P. maculata*. Although it is an annual species, it normally grows to a maximum of 100mm and would not normally die at 50mm.

Pathways for toxin accumulation in *P.maculata* not presently know. Literature reviews to continue tomorrow. Further testing of sea slug gut contents planned with Cawthron through next week

Appendices
Appendix 1 Field Report , Appendix 2 Identification, Appendix 3 Organism Profile

3 Report from MAF National Centre for Disease Investigation

Operations Report

- The area involved is different for the different species, dogs have been affected on a range of beaches in the Auckland and Coromandel regions. Penguins have been reported dead in the Far North, Rodney, Auckland, Coromandel and Bay of Plenty regions. Fish have been reported dead in the Far North and Auckland regions. Dolphins were found in the northern parts of the Hauraki Gulf and one at Waiheke Island.
- There have been 99 reports of dead penguins on beaches.
- There are 8 reports of dead dolphins being found in the Hauraki Gulf since the 9th July
- There are 37 reports of sick dogs, of which at this stage 18 are considered cases
- Of the dog case reports 3 have died and 3 have been euthanized due to their condition.
- There are two clinical syndromes observed in dogs, one is a sudden onset of repeated vomiting at the beach followed by death within hours or rapid recovery, the other is the onset of neurological symptoms including seizures, unusual behaviour and altered mental status within hours of being at the beach and either gradual recovery or gradual deterioration.

Appendices
Appendix 4 Maps of the report cases

4 Report from the National Institute of Water and Atmospheric Research

NASA Ocean colour imagery of Hauraki Gulf, July – August 2009

Data contact: J.N. Schwarz and H. Chang, NIWA funded under FRST Coasts & Oceans OBI. j.schwarz@niwa.co.nz; h.chang@niwa.co.nz

Precis: Chlorophyll absorbs blue light strongly, but not green light.
The ratio of blue to green reflectance therefore provides an indicator for chlorophyll biomass. Quantitative values are derived using an empirical algorithm developed using thousands of data points from open ocean waters around the globe. This algorithm is compromised if substances other than chlorophyll are present and absorbing or scattering strongly in the blue-green – this includes dissolved organic matter, e.g. land run-off, and suspended sediments. In the central Hauraki Gulf, the water generally appears to be dominated by chlorophyll (M.H. Pinkerton) but near-shore, the chlorophyll satellite product is almost certainly in error. Near-shore phytoplankton blooms may be distinguished using a time record of several weeks, during which chlorophyll levels increase steadily and then decline. Any chlorophyll signal which appears over a shorter time-scale, such as days, is likely to be caused by sediments washing in from rivers or resuspended by wind mixing. The data provided

here were measured, processed and plotted by the Ocean Colour Biology Group at NASA and provided to Hoe Chang as a free, public domain data service.

Devonport: There's not much clear water between Devonport and the islands, the area is largely masked as being contaminated by reflectance from the land; Infer chlorophyll distributions from the clearer waters off-shore.

Evidence for a toxic bloom: We expect a toxic bloom to originate with the intrusion of warm water from off-shore (H. Chang): there is no evidence for such an intrusion in the coarse-resolution sea surface temperature imagery (attached).

There is evidence of bloom-level chlorophyll concentrations in the inner Hauraki Gulf, especially in the first 2 weeks of July (~4th to 17th). However, the chlorophyll concentrations do not appear to be as high as in previous confirmed Karenia spp. outbreaks (H. Chang). It is possible that higher spatial resolution data, with no spatial averaging, will show higher chlorophyll concentrations with more detail near-shore.

Later in July, distributions are much patchier in time and it is not clear whether sediments or chlorophyll is causing the elevated 'chlorophyll' product signal.
Given reports of fish-kills over the weekend, plus the history of toxic blooms in the Gulf, H. Chang believes that *if* a toxic bloom occurred, then it would have been Karenia spp. However, Hoe would expect chlorophyll concentrations > 10 mg m-3 to be found at the time of the bloom, and Karenia should still be present at detectable concentrations for weeks to months after the bloom. At this time, neither of these criteria appears to have been met.

We are now acquiring higher spatial resolution (250m c.f. 9km) satellite data for a more in depth analysis of chlorophyll and SST.

Dr Hoe Chang is now looking at samples collected by NIWA-Auckland over the last weekend.

5 Report from the New Zealand Centre for Conservation Medicine

We have received five penguins to date, three of which were too autolysed for examination. The two we necropsied were both in very poor body condition and the cause of starvation is not established but I guess these deaths are unlikely to be connected with the die off.

Have received histology results for the first one so far and, while the tissues looked in reasonably fresh condition, histologically it turned out they were too autolysed for any meaningful examination. Hopefully the second one will be better.

Appendices
Appendix 5 Pathology report penguins

6 Report from the Cawthron Institute

Update on testing of samples from Auckland dog death incident.

Samples (see table 1 and 2) received for testing have given the following results:

Algal analysis – No significant finds in either dog or environmental samples, including seaweed that was washed.

Algal toxin testing –Testing of the toxic *P.maculata* for the following toxins showed that none were present.

Domoic acid, Gymnodimine, 13-desmethyl-spirolide C, 13-desmethyl-spirolide D, Pinnatoxin A, Pinnatoxin D, Pinnatoxin E, Pinnatoxin F, Pinnatoxin G, Brevetoxin-B2, S-desoxy-brevetoxin-B2, Azaspiracid 1, Azaspiracid 2, Azaspiracid 3, Pectenotoxin 1, Pectenotoxin 2, Pectenotoxin 6, Pectenotoxin 11, Pectenotoxin 2 seco acid, Okadaic acid, Dinophysis toxin 1, Dinophysis toxin 2, Yessotoxin, 45-hydroxy-yessotoxin, Homoyessotoxin, 45-hydroxy-homoyessotoxin, anatoxin-a and Saxitoxins by immunoaffinity.

In addition a Phomidium-like species was detected in an algal sample collected from Narrowneck Beach and this was tested for anatoxin – a. none found.

The toxic *P. maculata* was also tested for PbTx-2 and PbTx-3 – none found.

Toxicity testing by mouse bioassay – Testing was completed on the samples shown in bold in tables 1 and 2. This showed that the sea slug sample was very toxic.

Species Identification – The sea slug sample was tentatively identified from a sample in poor condition as *P. maculata*. This was confirmed when an ethanol preserved sample was submitted,.

Other toxin testing – Based on some preliminary data we checked the *P. maculata* for tetrodotoxin (TTX). This was confirmed by liquid chromatography mass spectrometry (LC-MS). The dog stomach contents was also found to contain TTX. Samples test to date are highlighted in tables 1 and 2, except for the sea slug and stomch content sample all are negative. The Stomach content contained 2% (by weight) of the TTX found in the sea slug. The second sea slug sample (from Cheltenham beach) contained 20% of the TTX found in the first sea slug sample.

Appendices

Appendix 6 Samples at Cawthron Institute

7 Appendix 1 Field report – Auckland Regional Council

Pleurobranchaea sea slug collection, Narrowneck Beach, 12/08/2009

On the afternoon of 12/08/2009 several teams of volunteers from ARC, ACC, NSCC and DoC were deployed onto North Shore and East Auckland beaches to search for the sea slug *Pleurobranchaea maculata*.

At most beaches the area from the high tide line down to the bottom of the low tide area was surveyed. This included the main beach as well as any rock pool or rocky shelf areas.

The locations surveyed were:

North Shore

- Long Bay
- Brown's Bay
- Milford Beach from Tiri Road to Wairau Outlet
- Castor Bay
- Takapuna Beach (only down to just below mid tide)
- Narrowneck Beach
- Cheltenham Beach

Tamaki Estuary / East Auckland

- Mt Wellington war memorial Reserve area
- Eastern Beach (high tide area only)

About 120-150 *Pleurobranchaea* were found at Narrowneck Beach and a single specimen at Cheltenham Beach. No *Pleurobranchaea* were found at any of the other sites. We believe the bulk of the animals present along the length of Narrowneck Beach were collected.

The animals were all found in the area of the beach between mid and low tide amongst clumps of seaweed (predominantly *Carpophyllum maschalocarpum* and some *Hormosira banksii*) that were washed up on the shore (see photos 1 and 2, further photos and video can be found here).

The bulk of the animals were found dead, with only 4 larger individuals found alive. Dead animals were opaque with little colouring (see Photo 3) while live animals still retained their characteristic brown speckling (see Photos 4 and 5). The animals ranged in size from about 30mm to 80mm with the bulk in the 30-50mm range.

The bulk of the *Pleurobranchaea* were frozen on the evening of the 12/08/09 and flown to Cawthron Institute for toxicity testing on the morning of 13/08/09. Three representative animals were also sent to Cawthron preserved in ethanol for identification purposes.

Three live animals and 6 representative dead animals were delivered to Margaret Morley for identification and inspection. The three live animals may be preserved for the museum collection but the six dead animals will be frozen once inspected and can be made available to Cawthron if they require more material.

Of interest was that many of the dead animals collected had eviscerated their gut contents. Furthermore, about 8 individuals gut contents appeared to contain Coralline red algae (Photo 6) but no other identifiable material. Margaret Morley speculates that the Coralline algae may have been inadvertently ingested when the *Pleurobranchaea* were consuming marine worms known to live within Coralline turf.

Photo 1: Area of Narrowneck Beach where most animals were found (low tide)

8 Appendix 2 Identification report – Margaret Morley

The photos shown to me by Hazel on 12 Aug 2009 are of *Pleurobranchaea maculata.*

Pleurobranchaea maculata (Quoy and Gaimard, 1832) Family Pleurobranchidae Photograph and text in Photographic guide to NZ Seashells by Morley and Anderson 2004 New Holland p122.

Common name Grey side-gilled sea slug.

Size Adult 80-100 mm

Identifying features Soft sea slug, no shell. Body smooth to touch but covered with minute puckers and folds. Colour pale grey, densely patterned with short, brownish-black lines. The feathery gill is partly hidden under the right side and extends further when the animal gets stressed. The rhinopores are widely placed on each side of the head.

Range North and South Islands.

Habitat Lives in all habitats from intertidally in harbours and to depths of 250 m off open rocky coasts. It is not usually common but opisthobranchs well known to appear either in big numbers or be absent or rare.

Diet Fast, active hunters **e**ating sea anemones, marine worms and molluscs. No reference in literature to eating algae or cyanobacteria, however the skin secretes a strong acid on disturbance.

Personal Records
13 Jul 1991 Over 30 of this species at extreme low tide at Bucklands Beach where they had come in to spawn. The spawn is laid in a spiral coil.
1 July 1991 Kohimarama 1specimen
19 Dec 1991 Bay of Islands subtidal 1 specimen
15 May 1991 Oneroa Waiheke 4 specimens, snorkel.
1 Aug 1992 Mathesons Bay, Leigh, low tide. 1 specimen.
10 Mar 2004 Whitianga, intertidal attached to *Hormosira banksii.* 1 specimen
10 Mar 2004, 19 Sep2004 Karaka Bay, Tamaki Estuary. Low tide rock platform.

Auckland Museum has more records.
ARC publications by Hayward and Morley on Waitemata Harbour, Tamaki Estuary and Waitakere coast have species lists showing locations for all species found. Bruce also has unpublished data from our ongoing studies in the Hauraki Gulf which may assist.

Opisthobranchia experts- Drs Michael Miller (Auckland) and Richard Willan (Darwin).

9 Appendix 3 Organism profile

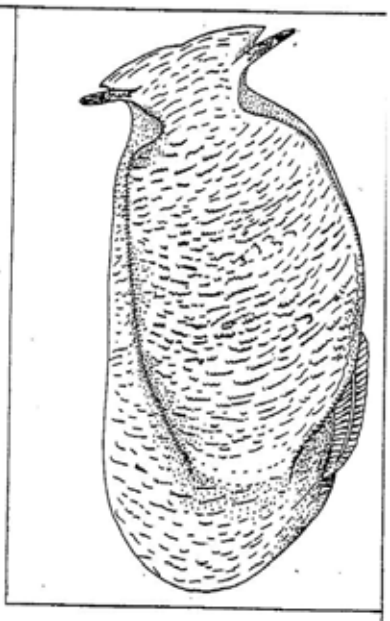
Pleurobranchaea maculata (Quoy & Gaimard, 1832)

AWBP : 282, pl. 51 No. 3; M&M : pl, 11 No. 81 (as *Pleurobranchaea novaezelandiae*); Willan 1975; Willan 1982; Ottaway 1977b

DESCRIPTION:

Distinguished from the other New Zealand pleurobranchs by the absence of a shell, the smaller extent of the mantle and its fusion with the foot in front and the wide separation of the rhinophores.

Animal large and smooth to the touch, but covered with minute puckers and folds. Colour pale grey, densely patterned with meandering broken lines of dark greyish-brown. Foot projects a considerable distance behind the mantle. Radula with a central tooth, and about 40 to 50 laterals most of which have an accessory spike. Jaws composed of numerous, small rounded or polygonal, interlocking elements all of which bear small denticles along their anterior margin. *Pleurobranchaea granulosa* is another synonym in New Zealand literature.



SIZE: 100 mm

HABITAT: The most catholic of all opisthobranchs regarding habitat. *P. maculata* occurs on all substrates from silt of harbours to rocky substrates of open coasts. It is even known from diatomaceous oozes on the continental slope.

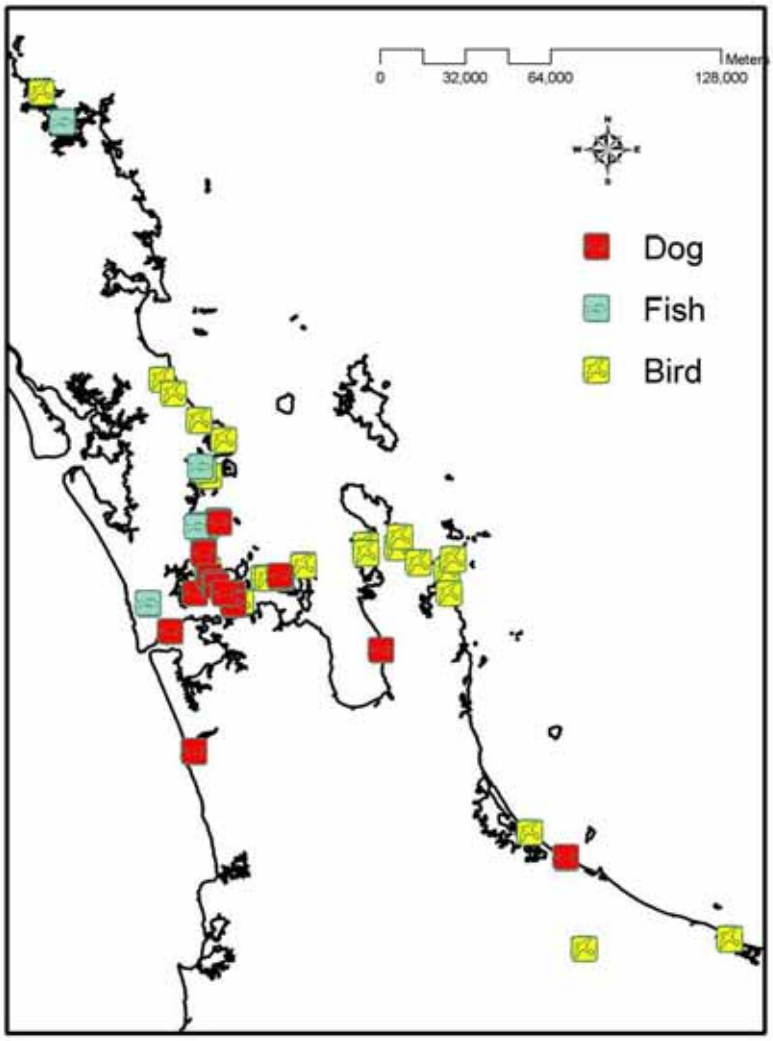
RANGE: Throughout New Zealand. Depth range - intertidal to at least 250 metres. Elsewhere *P. maculata* is widespread in temperate waters of the Indian and Pacific Oceans.

BIOLOGY & ECOLOGY:

Described by Cheeseman (1878) as *P. noveazealandiae* (and illustrated by Miss E. Cheeseman in the well known colour drawing--reproduced by Powell (1979 in plate 11 No.8)), this species has been satisfactorily identified by one of us (R.C.W.) with Quoy & Gaimard's original *P. maculata*. Willan (1975) has given a full account of its anatomy and ecology.

Pleurobranchaea maculata has been found to be an opportunistic feeder on soft-bodied invertebrates, especially sea anemones and mobile annelids and molluscs. Willan (1975) conducted experiments on food attractiveness and preference, and found the anemones *Anthopleura aureoradiata*, *Isactinia olivacea* and *Anthothoe albocincta* to be preferred in that order. On the basis of laboratory experiments, Ottaway (1977b) concluded *P. maculata* might be determining the lower limit for growth of the anemone *Actinia tenebrosa* by eating all those individuals that occur below E.L.W.N. level.

10 Appendix 4 Maps of report cases



Situation Report
Animals affected on beaches, Auckland

Date Thursday 13 August 2009

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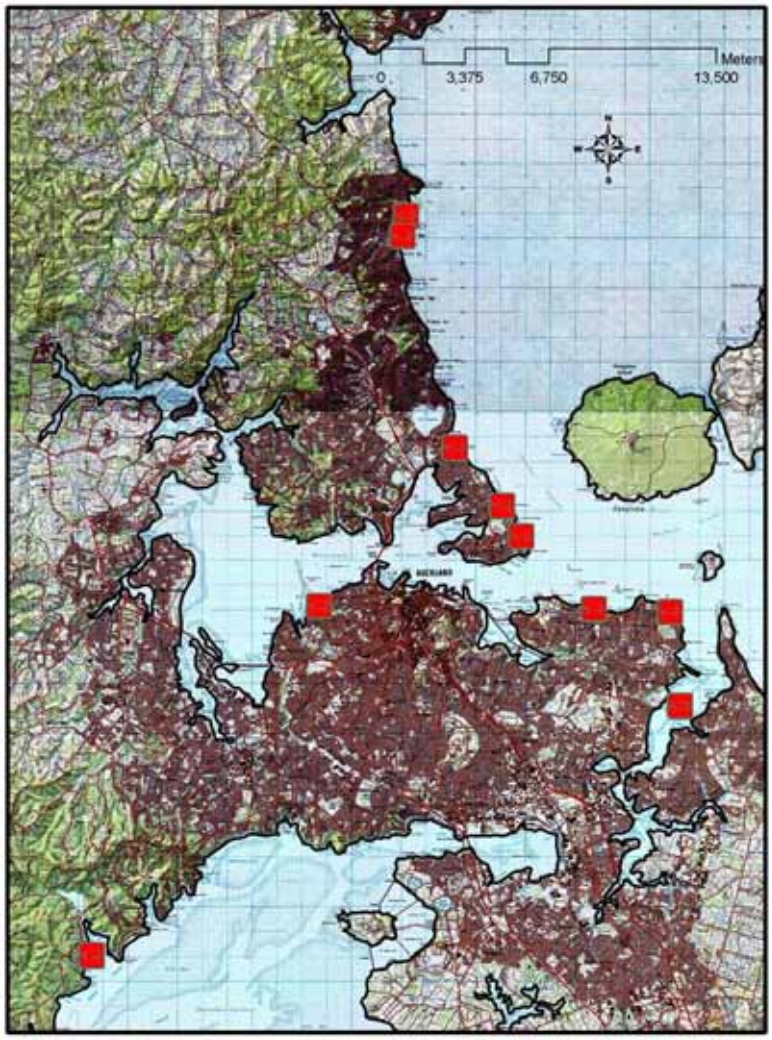
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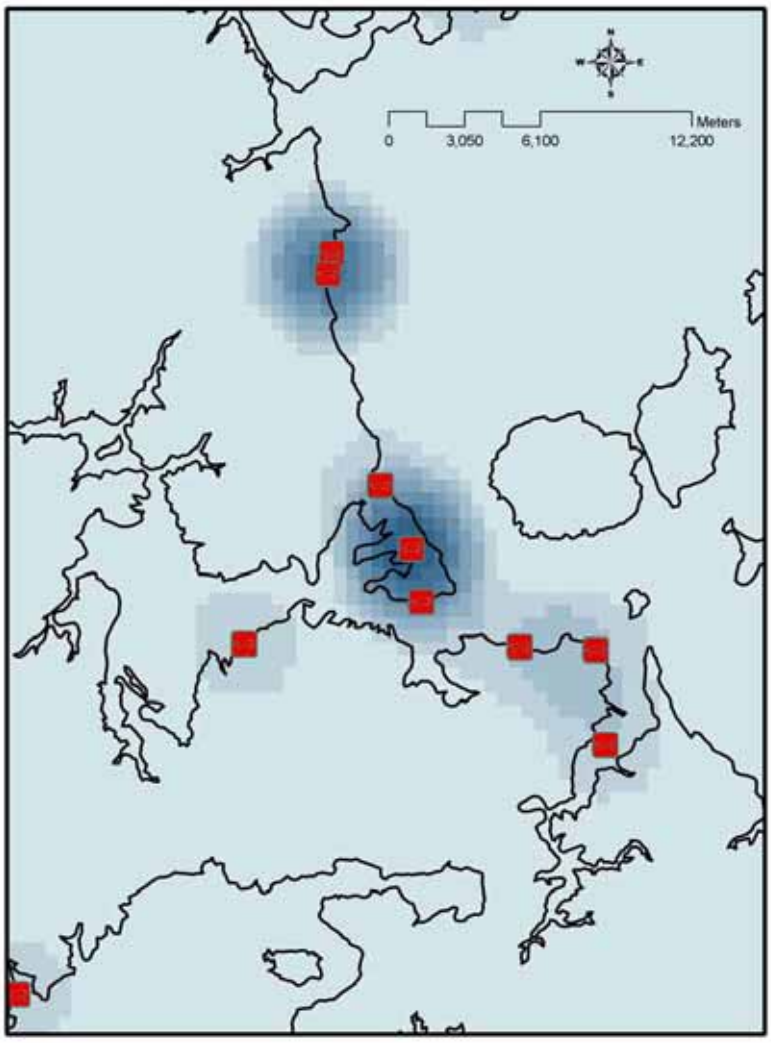
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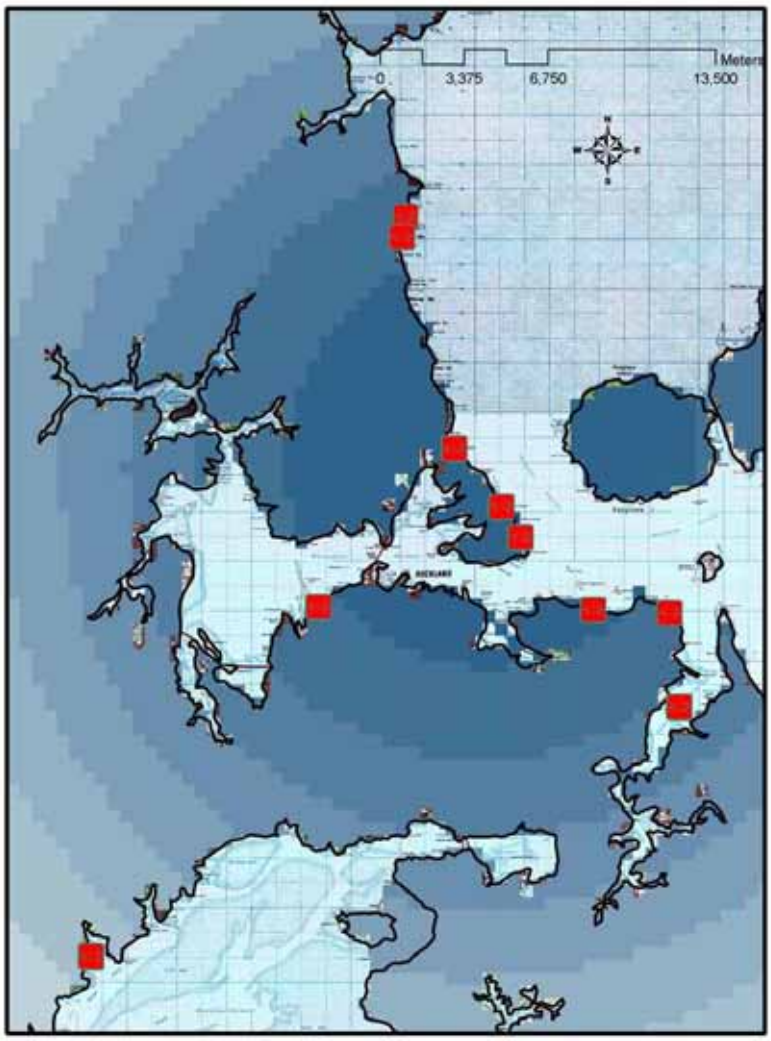
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Meters

11 Appendix 5 Pathology report - Penguins

PATHOLOGY REPORT

TO: Dr. Caleb King, Investigation and Diagnostic Centre, Biosecurity New Zealand.

Gribbles Accession #: AU0917212 Date Sent: 13/8/09:

Type: Avian Sex: F Age: Unknown Species: Little Blue Penguin

ID: #1 At Risk: Unknown Affected: Unknown

Owner: MAF:BNZ Previous Accession: N/A Type: Post Mortem

HISTORY
Penguin carcase recovered by Navy divers off the East coast of Great Barrier Island 7/8/09 and brought to the NZCCM on 8/8/09 for gross necropsy. Bird submitted as part of an investigation by MAF:BNZ of a bird die-off in the Hauraki gulf (Investigation 2211)

GROSS NECROPSY FINDINGS
Body condition emaciated – no subcutaneous fat. Eyes sunken/concave; Body very wet/water-logged;
No external lesions other than moderate abrasions next to left eye (suspect PM scavenging as no associated haemorrhage).
Weight: 623gms (wet)
Tarsus 28.21mm; Bill 26.5mm; Wing 106.8mm
Respiratory system: Lungs congested, small amount of free blood in air sacs.
Cardiovascular system: No significant findings; spleen autolysed.
Digestive system: Ventriculus empty, liver grossly normal, oral cavity and intestinal tract normal, No internal parasites seen.
Urinary system: No sig findings
Reproductive system: Ovary appears normal
Endocrine system: No sig findings
Nervous system: No sig findings

DIAGNOSTIC SAMPLES COLLECTED

10% Formalin:
Brain, liver, kidney, ventriculus, heart, lungs

LABORATORY FINDINGS: See attached

POST MORTEM DIAGNOSIS
Open

LABORATORY FINDINGS: See attached

POST MORTEM DIAGNOSIS
Starvation, cause not established

Bethany Jackson BVSc
Medicine)
Intern in Conservation Medicine

Richard Jakob-Hoff BVMS, MACVSc (Wildlife

Senior Veterinarian – Conservation and Research

ACCESSION AU0917212
REPORT HISTO
REPORTSTATUS FINAL
OWNER AUCKLAND ZOO
SUBREF PENGUIN
SPECIES Avian
BREED Penguin
SEX Female
AGE 0 UNKNOWN
SENT 7/08/2009 4:36:20 PM
RECEIVED 7/08/2009 4:36:20 PM
SIGNEDDATE 10/08/2009 7:12:46 PM
SUBMITTER John Potter
TECHNICIAN CHARVE
LABORATORY Gribbles Veterinary Pathology Ltd- Auckland
LABADDR1 37-41 Carbine Road
LABADDR2 Mt Wellington
REPORTFEE 102.40
@RESULTS
@COMMENTS

AU 09- 17212

Patient: Little Blue Penguin

Gross examination: multiple fixed tissues

Histopathology:
Brain, Liver, Kidney, Lung, heart, Ventriculus / Gizzard - All tissues are severely autolysed and contain post mortem mixed bacterial proliferation.

Morphologic Dx: Open

Comment:
The tissue were too autolyzed to be of diagnostic value.

Catherine Harvey
B.V.Sc, Diplomate ACVP, Registered Specialist in Veterinary Anatomic Pathology

HISTOLOGY performed at Gribbles Veterinary Pathology Ltd- Auckland
Reference Ranges and Method Reference will be supplied on request Testing Requested
1 x Histology Multiple/Necropsy tissues
1 x Histology Multiple/Necropsy tissues
@END

12 Appendix 6 Samples at Cawthron Institute

Table 1. Samples sent to Cawthron

Sample Type	Sender	Label	Date Sampled	Date Received
Stomach contents	Gribbles Veterinary, Auckland	Stomach Contents B248106 (Beagle, M, 1yr-9mths)	10 Jul 2009	14 Jul 2009
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit #1 B251889 first vomit	4 Aug 2009	5 Aug 2009
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit #2 B251889 second vomit	4 Aug 2009	5 Aug 2009
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit #3 7175313	3 Aug 2009	5 Aug 2009
Jelly fish	Ciaran Edwards (North Shore City)		3 Aug 2009	7 Aug 2009
Dried seaweed – Narrowneck Beach	Ciaran Edwards (North Shore City)		3 Aug 2009	4 Aug 2009
Damp seaweed – many types from Narrowneck Beach	Bill Trusewich (Department of Conservation)		4 Aug 2009	4 Aug 2009
Dead pilchards from Orewa Beach	Rodney DC	V637	Unknown, mid Jul	11 Aug 2009
Possible Pleurobranchea from Cheltenham Beach	Bill Trusewich DoC	V639	12 Aug 2009	13 Aug 2009
Possible Pleurobranchae from Narrow Neck	Marcus Cameron ARC – EtOH preserved	V640	12 Aug 2009	13 Aug 2009
Possible Pleurobranchae from Narrow Neck	Marcus Cameron ARC	V641	12 Aug 2009	13 Aug 2009

Table 2 Samples collected by Cawthron on the 7 August 2009

Sample Type	Sampling Location	Date Sampled	Date Received
Sediment/sand	Sand surface Cheltenham Beach	7 Aug 2009	10 Aug 2009
Seawater	Tamaki	7 Aug 2009	10 Aug 2009
Seawater	Narrowneck Beach, freshwater inlet	7 Aug 2009	10 Aug 2009
Seawater	Narrowneck Beach, freshwater inlet	7 Aug 2009	10 Aug 2009
Scraping (green)	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Limpets	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Mussel scraping	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Seaweed and sponge from high tide line	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Greenshell mussels	**Cheltenham Beach**	**7 Aug 2009**	**10 Aug 2009**
Algal mat	**Cheltenham Beach**	**7 Aug 2009**	**10 Aug 2009**
Sponge low tide	**Cheltenham Beach**	**7 Aug 2009**	**10 Aug 2009**
Three sea slugs (size)	**Narrowneck Beach**	**7 Aug 2009**	**10 Aug 2009**
Small unidentified sea slugs (10-15 mm long)	Narrowneck	7 Aug 2009	10 Aug 2009

13 Appendix 7 Mass spectrometric evidence of TTX.

Figure 1. LC-MS chromatogram a)pure TTX, b)dog stomach contents and c) sea slug.

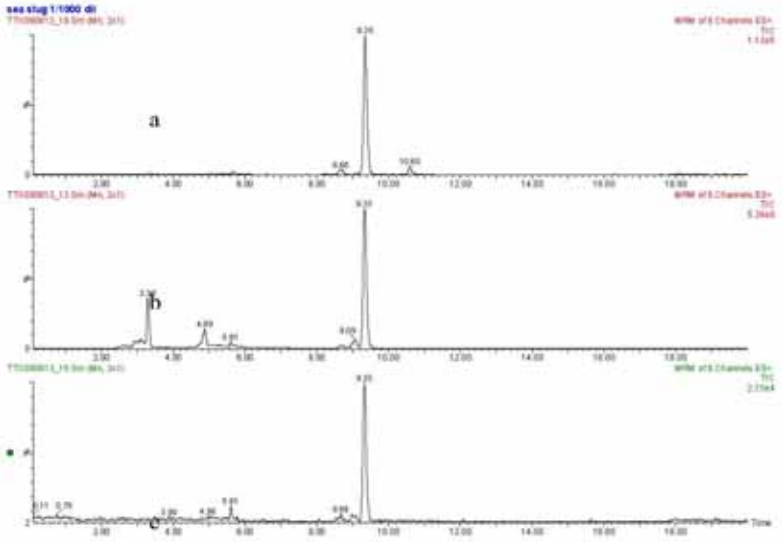


Figure 2. Daughter ion TIC. Sea slug top, pure TTX below

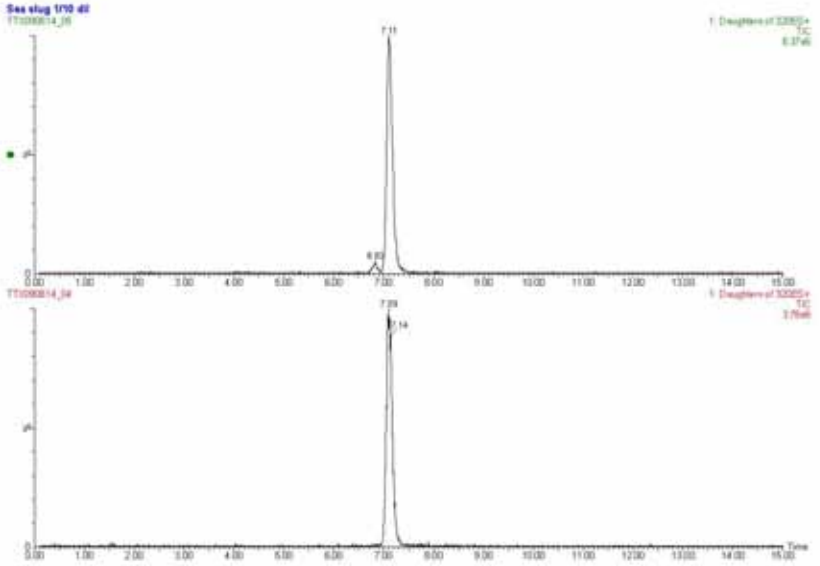
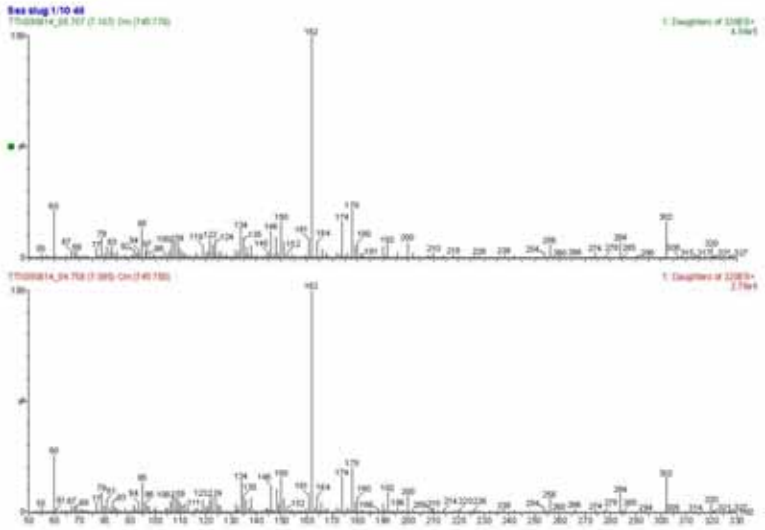


Figure 3. Daughter spectrum. Sea slug top, pure TTX below



14 Appendix 8 Case Reports of Poisoned dogs

	Reported	Detail of call	Place	Onset	Fate	Clinical signs
1	5/08/09	dog sudden death - pls call	Cheltenham Beach	01/07/09	Died	Vomiting, bradycardia
2	6/08/09	dog sick after beach, recovered	Narrowneck Beach, Devonport	07/07/09	Recovered	Vomiting within 15 minutes of beach, recovered after vet visit
3	7/08/09	dog sick after beach, death	Narrowneck Beach, Devonport	09/07/09	Died	Vomiting within 20 minutes of beach, vet visit, died within an hour of beach
4	6/08/09	Dog got sick 15 July after being on Eastern Beach. vomited, diarrhoea and sleepy - picked up couple days later	Eastern Beach, Howich area	15/07/09	Recovered	Vomited, diarrhea and sleepy - recovery over 2 days
5	5/08/09	Dog euth after uncontrollable seizures	Cox's Beach	25/07/09	Euthanized	Neurological signs
6	6/08/09	dog sick after beach, recovered	Browns Bay / Waiake Beach	25/07/09	Recovered	Neurological signs, gait change within 15 minutes of beach, recovered gradually
7	5/08/09	Dog poisoned angry about cost	Kohimarama Beach	27/07/09	?	
8	5/08/09	dog swam at Karaka Bay 29/30July. Hosp next 4 days at Kohimarama vets with unexplained illness.	Karaka Bay	29/07/09	Recovered	Neurological signs
9	5/08/09	dog sick, saw vet	Torbay area, Long Beach	30/07/09	Recovered	Gagging, diarrhoea, agitated within 4hrs of beach visit.

10	6/08/09	dog developed CNS signs 36 hrs post swimming at Browns Bay. Walking in cicles, disorientated.	Browns Beach	30/07/09	Recovered	Circling, disorientated, distressed. Visited vet. No diagnosis given
11	6/08/09	Sick dog 1 hr after swimming is sea	Waiomu Beach, North of Thames	01/08/09	Recovered	Vomiting within 1 hr, retching continuing for 36 hrs plus generally weak and shaky
12	6/08/09	Dog sick following walk near Browns Bay. Awaiting hx from vets.	Browns Bay	01/08/09	Recovered	Vomiting, hosp 3 days
13		dog sick after beach visit	Onetangi bay beach, Waiheke	02/08/09	Recovered	Neurological signs, cardiac signs and diarrhoea, gradual recovery 3ds
14		dog sick after beach, death	Narrowneck Beach, Devonport	03/08/09	Died	Vomiting within 15 minutes of beach, death 1 and half hours after beach
15		dog sick after beach, recovered	Narrowneck Beach, Devonport	03/08/09	Recovered	Vomiting within 10 minutes of beach, vet visit, likely to recover
16	6/08/09	dog lost near Tamaki esturary and found dead next morning	Manor Park Road, Tamaki estuary	06/08/09	Died	Found dead
17	13/08/09	Dog being euthanised - please ring to see if there is anything that can be done. Been on drip at vets since Saturday, been to North Shore beaches	Auckland		Euthanized	Neurological signs
18	13/08/09	CNS signs. At VSG being euthed today	Orewa beach and Milford-Takapuna Beach	02/08/09	Euthanized	Neurological signs, Muscle twitch that progressed to seizures over 2 days.

BIOSECURITY NEW ZEALAND

Animals affected on Auckland beaches, 13 August 2009
National Centre for Biosecurity and Infectious Disease

Ref:

SITUATION REPORT

Investigation: 2211 Dead dogs Auckland	**Initiated**: 3 August 2009
Date of Report: 24 August 2009	**ARC contact:** Grant Barnes
Date of Last Report: 13 August 2009	**Number: 3**

Table of Contents

1 Situation Report - Overview

Current Objectives :

1.1 Protect the public
1.2 Delimit the extent of the cases
1.3 Determine origin
1.4 Determine an appropriate response for the apparent risk

- The Auckland Regional Council are the lead agency for this response from the 10th August 2009 and are coordinating the technical, communications and management teams in an effective manner. Conference calls are on an as-required basis within each team and participants are kept informed at the major conference call twice each week.

- A technical advisory group consisting of ARC and MAF personnel met last week to consider the information to hand and look at appropriate strategies to answer the many questions raised by this new finding of a toxin in sea slugs. It is apparent that there are several science streams involved and answers to these questions will require significant resources and time to resolve.

- The incursion investigation team at the MAF National Centre for Biosecurity and Infectious Disease, have continued the surveillance and casing project for the dogs and penguin calls to the MAF 0800 80 99 66 free phone number that have been received since the public awareness was raised. A total of 303 calls have been received to date.

- The National Poisons Centre in Dunedin, Otago are continuing to provide expert advice on toxicology, to assist the response.

- The Cawthron Institute have confirmed that similar concentrations of tetrodotoxin (TTX) are in a grey-gilled sea slug found alive at Narrowneck beach. The testing of slugs from different beaches has shown variation in the concentration of TTX. The testing of porcupine fish and pilchards from the Long Bay region, has found them to be negative for TTX.

- The New Zealand Centre for Conservation Medicine has post mortemed six penguins to date. The gross post mortem results indicate that these birds are in poor body condition and starvation is the likely cause of death. Histology on two of these animals has not shown evidence of acute poisoning as the cause of death.

- Veterinarians in the Auckland region are continuing to phone the MAF 0800 80 99 66 phone number to report cases in dogs. There have been two reports of dogs with an acute onset of vomiting followed by ataxia, paresis and reduced mental arousal in the Waiomu Beach, Thames region.

- North Shore City Council has erected signage on the North Shore beaches to inform people not to take their dogs on the beach. Auckland City Council has erected signage on Eastern Suburb beaches.

- The public health warning messages remain in effect at this time.

2 Report from Auckland Regional Council

- The dog allegedly poisoned after swimming at Stanmore Bay died of natural causes. The marine specimens collected at Stanmore Bay where identified as parchment work cases and a sea cucumber (most likely *Stichopus mollis*). Both were negative for TTX.

- The porcupine fish collected 14/8 by Massey University and pilchards collected 28/7 from Long Bay were both negative for TTX.

- The limpets and little sea slugs (*Onchidella nigricans*) collected 7/8 from Narrowneck were both negative for TTX.

- The oyster sample collected 19/8 from Long Bay was negative for routine marine biotoxins, we are still awaiting TTX result.

- Another Pleurobanchaea was analysed from Cawthron's holding tank and found to the same TTX concentration as in the initial sample, this indicates the toxin is not depurating quickly and is not toxic to this organism.

- Several bacterium have been isolated from the gut of Pleurobanchaea; four of these have tested negative for TTX.

- Approximately 20 P.maculata were located at Narrowneck beach on 21/08; half were found on the lower beach in amongst seaweed, the other half were found underneath rocky platforms or in shallow rock pools. 90% of the specimens were large (~100mm). All specimens captured were alive and have been sent to Cawthron for testing.

Cawthron have run three batches of samples on the LC-MS for TTX.

1. **Batch one** - sea slug homogenate (collected 7/8/9 from NN), greenshell mussels from Cheltenham Beach, dried seaweed from Narrowneck, sponge from Cheltenham, an algal matt Cheltenham, Dog stomach contents (10 Jul 09) (George Dog Beagle, died after playing with something 'slimy' - Gribbles Ref: B248106), Pilchards from Orewa (sent from Rodney district Council, arranged by ARPH), sea slug from Cheltenham beach.

2. **Batch two** - MS experiments on sea slug to confirm identification and a sub sample of the frozen sea slugs collected in bulk lot (150-200 slugs) from Narrowneck (NN - 12/08/09) were dissected. Result; 82% of TTX was in the internals (stomach, hind gut etc), 15% in outside tissues, 3% in heart + gills.dissected sea slug (heart + gills, viscera + gonads and outside tissues)

3. **Batch three - brief summary:**

    - Sea slug collected from NN dead on capture (CAW ID V645) - 60% of TTX was in the internals, 30% in the outside tissues and 10% in the gonads.

- Live sea slug from Cheltenham (CAW ID V644) - levels of TTX were ~5 times lower than slugs from NN, 85% TTX in outside tissues, 12% in internals, 5% in gonads

- Live slug collected from Nelson 18/8 dissected same day (CAW ID V646) - Levels of TTX were ~1000 times lower than slugs from NN, 85% of TTX was in the outside tissue, 9% in hind gut, 5% in the mouth-stomach, 1% in gonad

- A whole live slug collected from NN (1 of the 5 collected 17/8 - CAW ID V645-2) was analysed and the levels of TTX were the same as in the previously tested dead slugs, this suggests that the toxin is most likely not responsible for the sea slug deaths. The remaining 4 slugs are in a tank and seem to be healthy, they are eating fish food and crawling around. The plan is to dissect another one tomorrow and see if toxin levels are going down. We have also collected faecal pellets for analysis. Bacteria from the viscera of sea slugs of been isolated and will be analysed for TTX.

- **Other samples:**

  - Two Porcupine fish were dissected, both stomachs contains fresh prey (lice in one and small gastropods in the other) which shows that they had recently feed before there deaths. One of the two fish had an enlarged discoloured liver, 5-10 time the size of the other, suggesting that it was not very healthy. A sample of each liver, gonads and rest of fish are being analysed today for TTX. Some pilchards were also sent in with these samples, they were collected 28th July.

  - Cawthron also received today the fresh rock oyster samples (n=18) from Long Bay. These will be analysed for the regulated marine biotoxins as well as TTX.

Surveillance

- ***Long bay:*** Collected live oysters on the rocks just out from the end of Beach Road. Very small specimens, collected an additional 8 animals to the 10 (18 total) to ensure a large enough sample was collected. No slugs were found in a search of the surrounding beach.

- ***Stanmore bay:*** Investigated reports of hundreds of slugs washed up on the western end of the beach. No slugs were found upon a thorough search of the algal wrack (reported location of slugs) and the surrounding beach. Possible mistaken items could have been parchment worm cases (Chaetopterous spp.). It was white, leathery and is broken up into slug shaped pieces. Furthermore it was scattered throughout the algal wrack. Chiton- one chiton was found washed up on the beach. Sea cucumber- Two sea cucumbers were found in the search, both white and resembling the slug in appearance. Ascidians- also slug-like (~4 were seen). Lots (~10-20) of dead eleven arm starfish were also washed up on the beach.

- ***Narrowneck Beach:*** Searched the beach ~1hr after low tide. Concentrated in the algal wrack in the same area as in Monday. No slugs found.

- ***Manukau Eastern Beaches:*** A search was underway today by MCC. Results pending.

Pluerobrachaea maculata

- Dr Richard Wilan, Curator of Molluscs at the Northern Territories Museum in Darwin, Australia further advises that…."*Pleurobranchaea maculata* can be found throughout the country, both intertidally and subtidally, often resting in depressions on the undersurfaces of stones. This species is tolerant of waters carrying suspended silt and thus appears in coastal situations such as harbours and estuaries where the other pleurobranchjs are not found. This behaviour is also correlated with the occurrence of food organisms; *Pleurobranchaesa maculata* is an opportunistic feeder and can take advantage of a wide range of prey species, whereas other New Zealand. Pleurobranchs feed on species of sponge which are themselves confined to clear water situations." Willan, R.C. 1984. A review of the diets in the Notaspidea (Mollusca: Opisthobranchia). Journal of the Malacological Society of Australia 6(3 & 4): pp125-142.

Allomycterus jaculiferus - "puffer fish"

- Regular reports are being received of stranded puffer fish. These are most likely an incorrect identification of the common porcupine fish Allomycterus jaculiferus. As noted above tests of the flesh, livers and gonads of two specimens of porcupine fish for TTX is presently underway by Cawthron. The sharp-nosed puffer fish Canthigaster callisterna, aka clown toado is rare in temperate waters and not usually found stranded on northern north Island beaches.

3 Report from MAF National Centre for Disease Investigation

Operations Report

- There have been 139 reports of dead penguins on beaches. There are two factors which suggest that the deaths in penguins are not related to the deaths in dogs. The widespread geographic distribution of the penguin deaths from Northland to the Bay of Plenty and the post mortem results indicating starvation as the cause of death.

- There are 8 reports of dead dolphins being found in the Hauraki Gulf since the 9th July. Three of these animals were washed up on beaches in the Warkworth region. The others are reported as Circular Bay, Kawau, Shakespear Regional Park, Motutapu Island, and Tiritiri Matangi Island.

- There are 41 reports of sick dogs, of which at this stage 18 are considered possible cases. Of the dog case reports 4 have died and 1 was euthanized due to it's condition.

- There are two clinical syndromes observed in dogs, one is a sudden onset of repeated vomiting at the beach followed by death within hours or rapid recovery, the other is the onset of neurological symptoms including seizures, unusual behaviour and altered mental status within hours of being at the beach and either gradual recovery or gradual deterioration.

- The last report we received of pilchards washing ashore in unusually high numbers was around the 31 July 2009 in the Long Bay area. Reports of other fish species washing ashore dead have also been received around Whangaparaoa (e.g. pufferfish, snapper) but not in high enough numbers to be considered significant therefore we consider these reports incidental.

- Most of the pilchards that have been examined using histopathology so far were too autolysed to provide much information on their cause of death. This is not uncommon in fish, which start decomposing quickly after death despite their otherwise "fresh" appearance. While a large number of frozen pilchards have been offered to us by members of the public who originally collected them for bait, these are generally unsuitable for histopathology due to freeze artefacts in the tissues. DNA has been extracted from these samples and are awaiting to be run on a PCR for pilchard herpesvirus. While we have the primers for this PCR, we are awaiting positive control material to arrive from Australia. It's hoped that this will arrive sometime next week. If this arrives as expected, results should be available later the following week.

- The pattern of appearance of the dead pilchards is similar to the pilchard mortality event that occurred in the same region (and subsequently nationwide) in June 1995, which was attributed to pilchard herpesvirus. Dr Rissa Williams looked at the water testing results for biotoxins around the same time of the mortality and there did not appear to be any problem phytoplankton species at a level that would cause the widespread pilchard mortality reported. I would also expect more marine species to be affected (in large numbers) if there was some sort of toxin or pollution event occurring - the fact it is species specific indicates it is more likely to be a disease.

Appendices
Appendix 1 Maps of the report cases
Appendix 8 Case reports of poisoned dogs
Appendix 9 Reports to the MAF 0800 80 99 66 number

4 Report from Leo Schep – toxicologist

- The text - Advances in Food and Nutrition Research Vol. 52 has a section of Tetrodotoxin Poisoning; it lists all species (crossing various classes) that have tested positive for tetrodotoxin (TTX); these include 8 species from the Gastropoda class. Thus it is possible this sea slug may have picked it up from feeding on other species known (or unknown as yet) to contain TTX. Note, only one gastropod species in this list had evidence of TTX in the whole body.

- Rates of elimination of TTX from animals (half life) can vary from 30 minutes to 3 - 4 hours, however, due to the rapid onset and severity of symptoms, toxicity can be fatal in short time intervals before it can be eliminated from the body (depending of course on the ingested dose).

Appendix 6 Worldwide species with tetrodotoxin
Appendix 7 Tetrodotoxin – brief summary

5 Report from the Cawthron Institute

The Cawthron Institute has analysed a dog stomach and vomit contents from three animals that had died after consuming an unknown material on several North Shore beaches in August 2009. The dogs were reported to experience the sudden onset of repeated vomiting at the beach followed by death within hours or rapid recovery; or the onset of neurological symptoms including seizures, unusual behaviour and altered mental status within hours of being at the beach and either a gradual recovery or deterioration.

Analysis of the dog stomach and vomit samples revealed no algal material and in response to further incidents of sick dogs and reports of dead penguins and pilchards, two Cawthron researchers travelled to Auckland on Friday August 7th 2009. The scientist visited Narrowneck, Cheltenham and Tamaki beaches and collected 13 samples and deployed solid phase adsorption toxin tracking (SPATT) bags. SPATT involves suspending in the water body small bags containing absorption substrates which accumulate toxins, thus providing a method of assessing extra-cellular toxins over an extended period of time. This new monitoring tool simulates the biotoxin contamination of filter feeding bivalves.

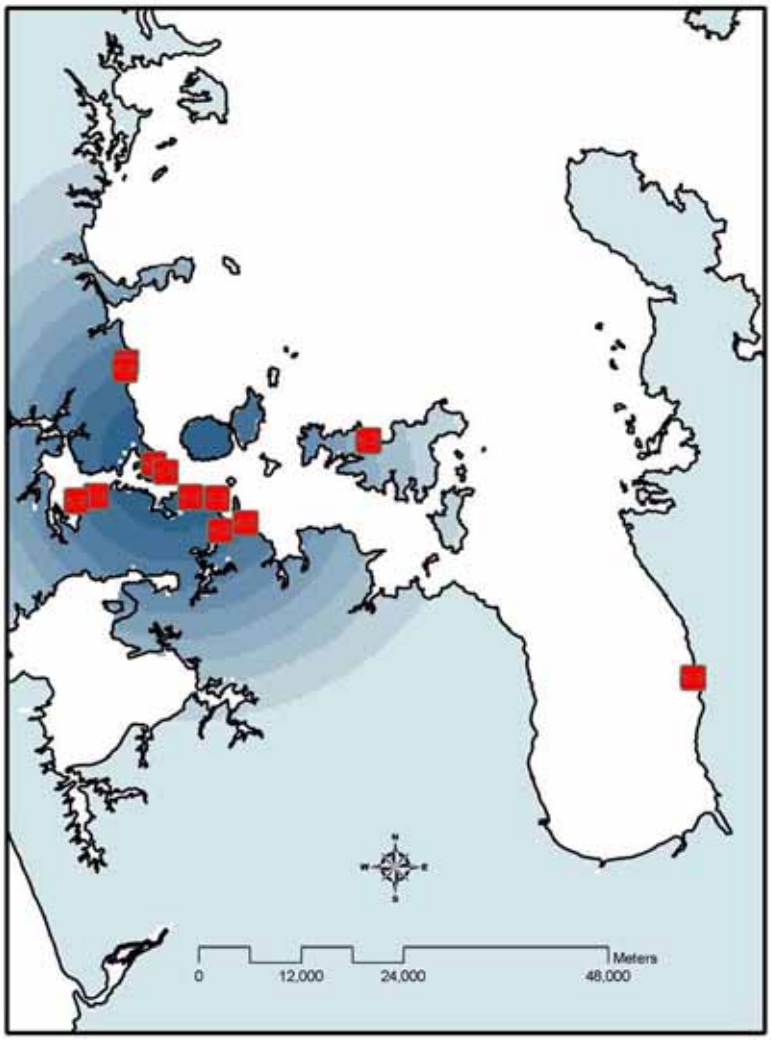
In addition to these samples, samples were sent to Cawthron by the Department of Conservation and North Shore City. This work was funded by the Cawthron Institute under the Seafood Safety programme (CAWX0307) and resulted in the identification of a toxic sea slug and the identification of the neurotoxin Tetrodotoxin (TTX) in the sea slug.

Further testing has been commissioned by the Auckland Regional Council (ARC) to confirm the presence of TTX and test more samples for TTX in addition to testing other samples for a range of toxins.

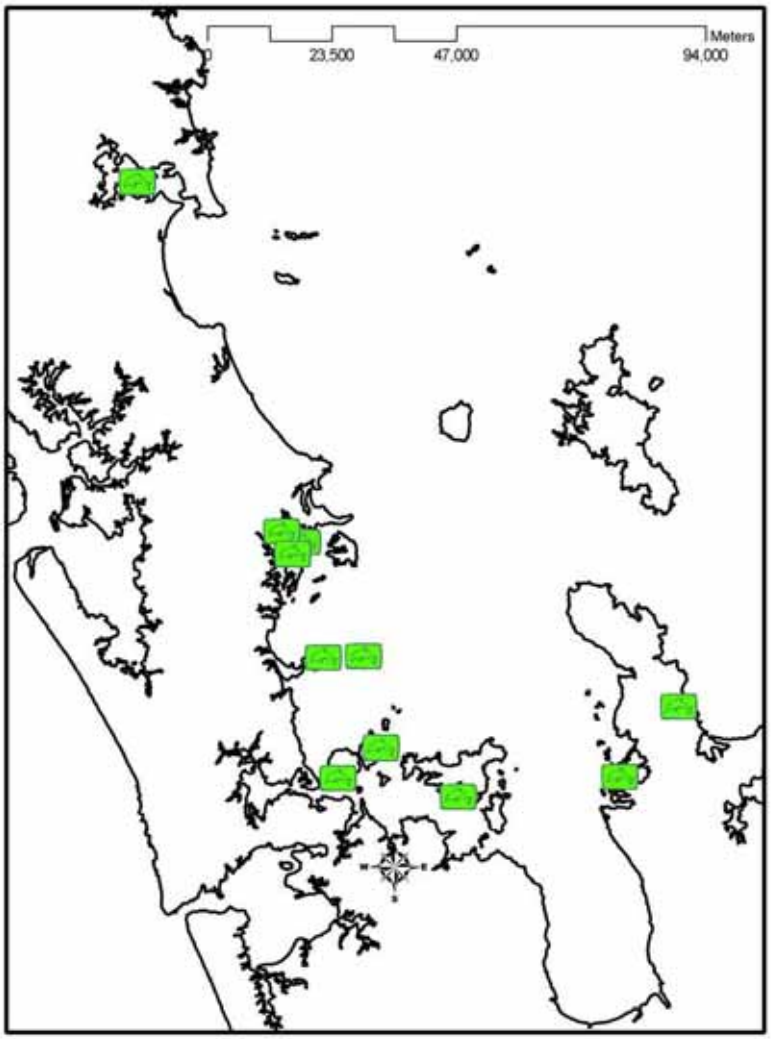
Appendices
Appendix 2 Microscopic analysis at Cawthron Institute
Appendix 3 Toxicology of methanol extracts
Appendix 4 LCMS analysis for lipophillic toxins
Appendix 5 Tetrodotoxin testing

6 Appendix 1 Map of the report cases

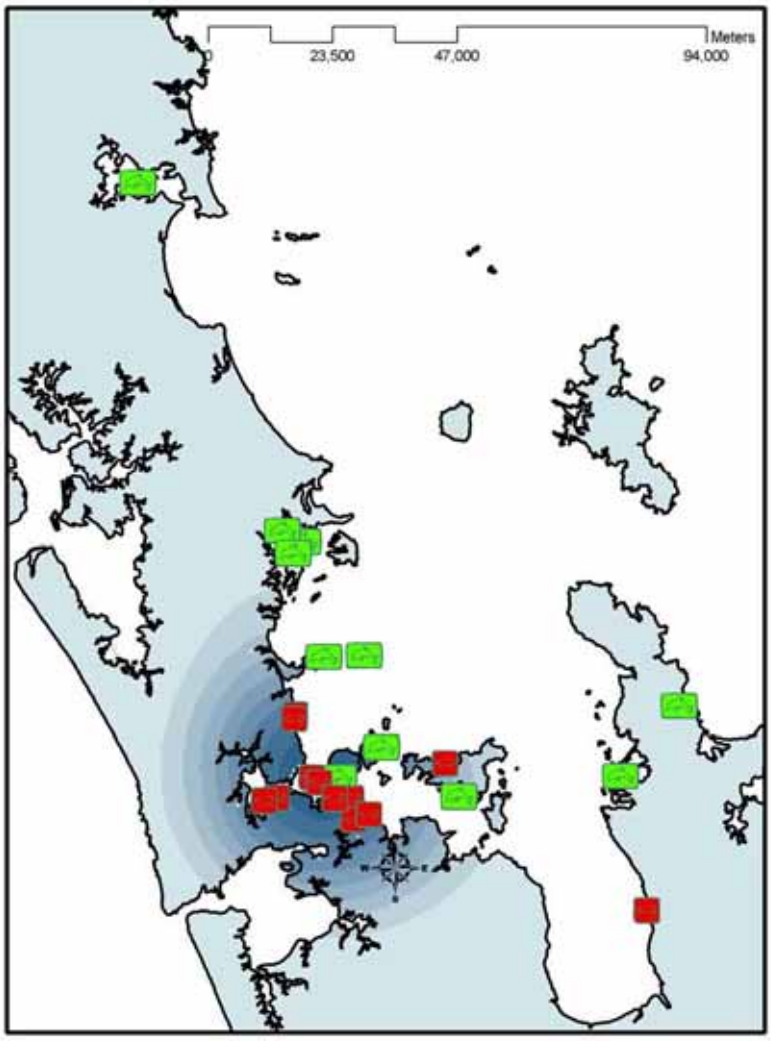
Dogs



Dolphins



Dolphins and Dogs



7 Appendix 2 Microscopic Analysis - Cawthron

Sub-samples of the dog stomach contents and vomit were taken and approximately 5 mL were used for species identification and enumeration using an inverted Olympus microscope (CKX41) and Utermöhl settling chambers (Utermöhl 1958). Seaweed samples were washed in freshwater and three 10 mL sub-samples were taken for species identification as described above. Seaweed samples were also checked for the presence of epiphytic species.

Sample type and location	Algae/cyanobacteria identified in sample
Stomach contents	No algal/cyanobacterial material observed
Dog vomit #1 B251889	Small diatoms present
Dog vomit #2 B251889	No algal/cyanobacterial material observed
Dog vomit #3 7175313	No algal/cyanobacterial material observed
Dried seaweed – Narrowneck Beach	No potentially toxic cyanobacteria/algae observed. A mixture of algal species present all in low abundance
Damp seaweed – many types from Narrowneck Beach	No potentially toxic cyanobacteria/algae observed. A mixture of non-toxic algal species present all in low abundance.
Algal mat, Cheltenham Beach+	Sheathed diatoms (*Naviculoid* spp.)
Sediment/sand, Cheltenham Beach+	Mixed diatoms and *Prorocentrum* cf. *triestinum**.
Seawater, Tamaki+	Debris
Seawater, Narrowneck Beach freshwater inlet+	Debris
Seawater, Narrowneck Beach freshwater inlet+	Debris
Scraping, Narrowneck Beach green+	Cyanobacterial mat - *Oscillatoriales (Phormidium* –like*)*

8 Appendix 3 Toxicology of methanol extracts

Samples were taken up in 1% Tween 60 in saline. Aliquots of the resulting solutions or suspensions were diluted to 1 ml in Tween-saline and injected intraperitoneally into Swiss albino mice, of body weight between 18 and 22 g. Although several samples affected the mice only one sample – sea slugs – exhibited significant toxicity.

Anatoxin analysis

A *Phomidium*-like species was detected in an algal sample collected from Narrowneck Beach. Freshwater species from this genera are known to produce the neurotoxins anatoxin-a and homoanatoxin-a (Wood et al. 2007). This sample was analysed for anatoxin-a and homo-anatoxin by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in Wood et al. 2007. No anatoxins were detected in this analysis (Limit of detection (LOD), 0.2 µg/L). A sub-sample of sea slug homogenate (2 g) was extracted with 18 mL of 90% methanol and the resulting extract analysed for anatoxins as described above, No anatoxins were detected in this sample (<10 µg/kg).

Lipophilic marine biotoxin analysis

A sub-sample of sea slug (2 g) was homogenated and extracted with 18 mL of 90% methanol. The methanolic extract was analysed for 26 marine biotoxins by LC-MS/MS (McNabb 2005). Results and LOD's are given in Table 4.

Saxitoxin analysis

The presence of saxitoxins was assessed in the sea slug sample using the Jellett Rapid PSP Test Kit (Jellett et al. 2002) according to the protocol supplied by the manufacturer. No toxins were detected.

Brevetoxin analysis

The presence of brevetoxins-2 and brevetoxins-3 was assessed in the sea slug sample using the methanol extract (see lipophilic toxin testing) using LC-MS (Cawthron method 40.106). No toxins were detected.

The results are presented in the table in appendix 4 below.

9 Appendix 4 LC-MS analysis for lipophilic toxins

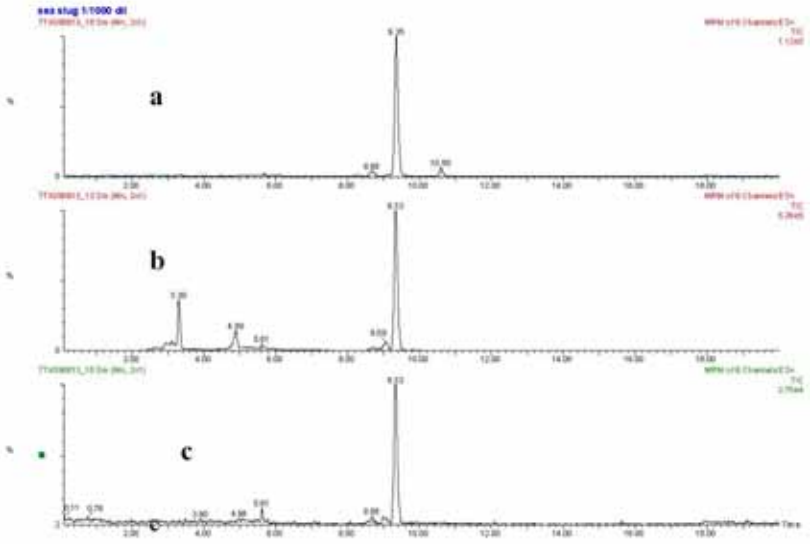
Toxin	Limit of detection (mg/kg)	Concentration of toxin detected (mg/kg)
Domoic acid	0.01	ND
Gymnodimine	0.05	ND
13-desmethyl-spirolide C	0.01	ND
13-desmethyl-spirolide D	0.01	ND
Pinnatoxin A	0.01	ND
Pinnatoxin D	0.01	ND
Pinnatoxin E	0.01	ND
Pinnatoxin F	0.01	ND
Pinnatoxin G	0.01	ND
Brevetoxin-B2	0.04	ND
S-desoxy-brevetoxin-B2	0.04	ND
Azaspiracid 1	0.01	ND
Azaspiracid 2	0.01	ND
Azaspiracid 3	0.01	ND
Pectenotoxin 1	0.01	ND
Pectenotoxin 2	0.01	ND
Pectenotoxin 6	0.01	ND
Pectenotoxin 11	0.01	ND
Pectenotoxin 2 seco acid	0.01	ND
Okadaic acid	0.01	ND
Dinophysis toxin 1	0.01	ND
Dinophysis toxin 2	0.01	ND
Yessotoxin	0.02	ND
45-hydroxy-yessotoxin	0.02	ND
Homoyessotoxin	0.02	ND
45-hydroxy-homoyessotoxin	0.02	ND

10 Appendix 5 Tetrodotoxin testing

Pure TTX was obtained as the kind gift of Dr. Munday. The TTX was sourced originally from Acros Organics (CAS 4368-28-9, citrate free, 1mg) and was dissolved in 10mL of 10% acetonitrile containing 0.1% formic acid. This solution was used for quantitative analysis after recalibrated using mouse bioassay that showed it was only approximately 0.25mg TTX, not 1mg as stated. All quantitative data in this report is based on this single TTX standard and therefore has significant uncertainty when comparing to other reported concentrations, the relative amounts are accurate.

A method was setup utilising a Waters Acquity™ UPLC and Waters Premier™ triple quadrupole mass spectrometer using a TosoHaas (Japan) TSK-GEL amide 80, 5µ, 2.0 × 250 mm column for HPLC separation. An acetonitrile gradient elution was employed. The system was buffered with Formic acid 50mM and ammonium formate 3.3mM. Mass channels monitored were: 320.1>162.1, 320.1>60.0 as determined by infusion of pure TTX and fragmentation using argon. Additional mass channels were included for known TTX analogues: 318.1>162.1 (11-oxo TTX), 304.1>162.1 (11-deoxy TTX), 302.1>162.1 (anhydro TTX), 290.1>162.1 (11-nor TTX). Samples were extracted with 90% methanol, 2g plus 18mL (McNabb et al 2005).Figure 1 shows the presence of TTX in sea slug and dog stomach contents samples. The main peak in all traces is TTX. Low levels of 11-nor TTX were also found in sea slug and dog stomach contents samples.

Figure 1 LC-MS chromatogram a)pure TTX, b)dog stomach contents and c) sea slug.



Figures 2 and 3 show daughter ion spectrum collected using the HPLC method described but scanning for all the products produced from the 320.1 parent ion. This result clearly demonstrates the fact TTX is present in the sea slug sample.

Figure 2 Daughter ion TIC. Sea slug top, pure TTX below.

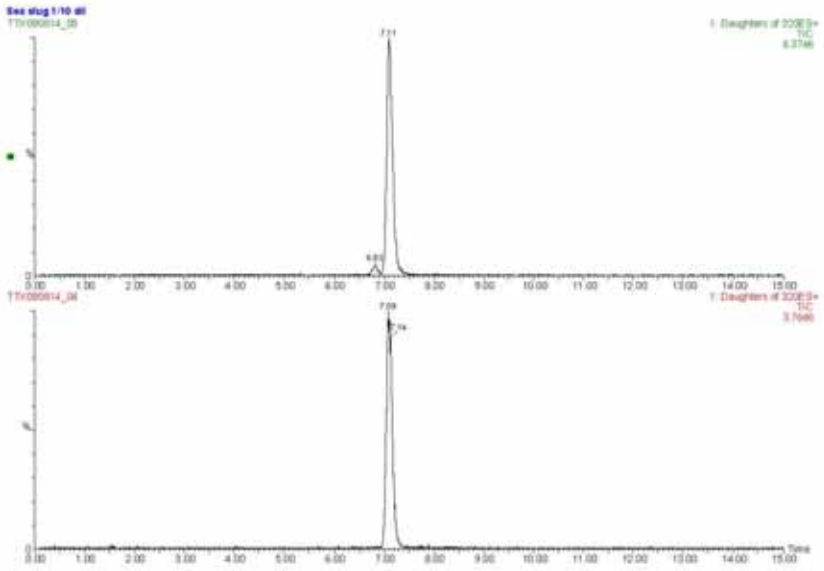
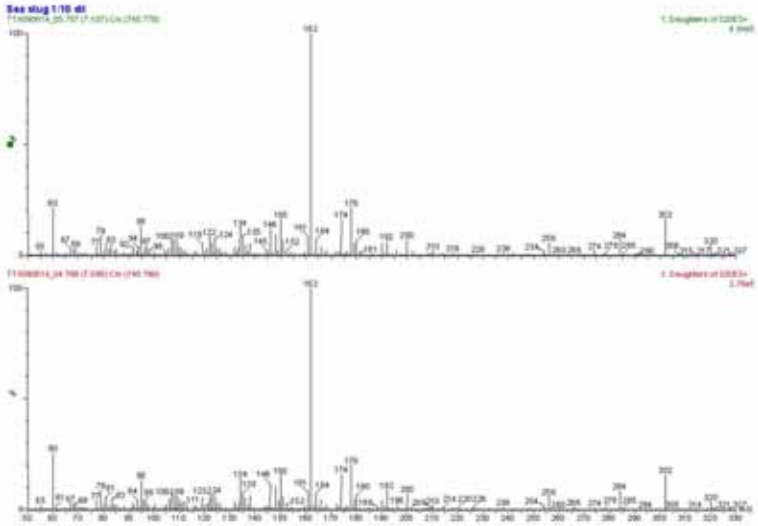


Figure 3 Daughter spectrum. Sea slug top, pure TTX below.



Additional confirmation information was gained from the ratio of the two TTX mass channels. In addition the 302.1 mass (nominal anhydro TTX) was formed by TTX "in source" and used to provide another mass ratio for confirmation. This data is presented in table 5 and adds additional weight to the absolute confirmation of TTX in the sample of sea slug and dog stomach contents.

Table 1 Ion ratio data for pure TTX, sea slug and dog stomach contents.

Sample	320.1>162.1/320.1>60.0	320.1>162.1/302.1>162.1
Pure TTX	4.2	5.8
Sea slug	4.5	6.3
Dog Stomach	4.4	6.1

Toxin mass balance calculation and estimation of TTX quantity

Additional mouse bioassay was completed on the sea slug sample and the same sample extract was also tested by LCMS. We also sent some of the pure TTX for mouse bioassay to confirm the concentration of the stock solution.

The additional mouse bioassay data confirmed a very high toxicity for the sea slug extract. The dog stomach contents were not assayed by mouse bioassay. Three sea slugs collected from Narrowneck Beach on the 7 August 2009 were homogenized. A sub-sample (20 g) of homogenate was extracted with methanol. The methanol was removed by rotary evaporation, the resulting residue was transferred with methanol to a 20 mL vial and methanol was removed under nitrogen. The sample was taken up in 1 ml of Tween-saline, and administered (various amounts) by intraperitoneal injection to 18-22g mice. The results are shown in table

Table 2 Mouse bioassay data for sea slug

Volume of extract (µL)	dilution	Sea slug dose (mg)	Death time
200	Neat	4000	< 1 minute
100	Neat	2000	< 1 minute
10	Neat	200	< 1 minute
10	1:10	20	< 1 minute
10	1:100	2	3 minutes
20	1:1000	0.4	9minutes
10	1:1000	0.2	9minutes
5	1:1000	0.1	survived

An approximate LD_{50} is 5-10 mg of sea slug per kg of mouse body weight (mg/kg).

Extracts were also sent for LCMS testing and were quantified using pure TTX. The extract contained 2.5 µg of TTX per mg of sea slug. As shown above the LD_{50} for sea slug is 5-10 mg/kg. A dose of 12.5 – 25 µg of TTX per kg is derived for the LD_{50} of pure TTX. If TTX is causing all of the observed toxicity this derived LD_{50} should approximately equal the LD_{50} of pure TTX. The experimentally derived LD_{50} of TTX is 15 µg/kg (Dr. Munday personal communication) thus proving that all the toxicity in the sea slug that we have observed is due to TTX and no other acute toxin is present.

Testing samples for TTX

Samples were prepared according to the method and tested for TTX. One frozen sea slug (collected by Cawthron 7 Aug 2009) was thawed and dissected into heart and gills, internal organs and the rest. The total TTX in each organ was calculated as follows: heart and lungs 2%, internal organs 87% and the rest 11%. The results of this testing are shown in Table 7.

Table 3 TTX testing results

Sample	Sample Description	TTX (mg/kg)
	Sea slugs collected by Cawthron 7/8/09	2450
B248106	Dog Stomach Contents	120
V638	Mussels collected by Cawthron 7/8/09	ND
	Sponge collected by Cawthron 7/8/09	ND
V637	Pilchards collected from Orewa beach	ND
B251889	Dog vomit aqueous fraction	ND
7175313	Dog vomit in sand	ND
	Algal mat collected by Cawthron 7/8/09	ND
V639	Sea slug (Cheltenham Beach)	930
V641	Dissected Sea Slug - Total	4437

The sample of sea slugs collected by Cawthron was extracted as described in section 3.1.2 for toxicological testing and the level of toxin recovered is likely to be lower than by the described method (2g plus 18mL 90% methanol).

11 Appendix 6 Worldwide Species with tetrodotoxin

Distribution of TTX in animals			
Hwang DF, Noguchi T. Tetrodotoxin poisoning. Advances in Food and Nutrition Research. 2007;52:141-236			
	Animals	Species	Part
1	Platyhelminthes	Flatworms	
	Turbellaria	*Planocera* spp.	Whole body
2	Nemertinea	Ribbon worms	Whole body
		Lineus fuscoviridis	Whole body
		Tubulanus punctatus	Whole body
		Cerebratulus lacteus	Whole body
		Cephalothrix linearis	Whole body
3	Mollusca	*Charonia sauliae*	Digestive gland
	Gastropoda	*Babylonia japonica*	Digestive gland
		Tutufa lissostoma	Digestive gland
		Zeuxis siquijorensis	Digestive gland
		Niotha clathrata	Digestive gland
		Natica lineata	Whole body
		Rapana spp.	Digestive gland
		Cymatium echo	Digestive gland
		Pugilina ternotona	Digestive gland
	Cephalopoda	*Hapalochlaena maculosa*	Postsalivary gland
4	Annelida	*Pseudopotamilla occelata*	Whole body
	Polychaeta	*Lepidonotus helotypus*	Whole body
		Halosydna brevisetosa	Whole body
		Harmothoe imbricata	Whole body
5	Arthropoda	*Atergatis floridus*	Whole body
		Zosimus aeneus	Whole body
		Carcinoscorpius rotundicauda	Egg
6	Chaetognatha	*Arrowworms*	
		Eukrohonia hamata	Head
		Parasagitta spp.	Head
		Flaccisagitta spp.	Head
7	Echinodermata	Starfish	
		Astropecten polyacanthus	Whole body
		Astropecten latespinosus	Whole body
		Astropecten scoparius	Whole body
8	Vertebrate	Takifugu spp.	Skin, liver, ovary
	Pisces	*Yongeichthys criniger*	Skin, viscera, gonad
	Amphibia	Taricha spp.	Skin, egg, ovary, muscle, blood
		Notophthalmus spp.	Skin, egg, ovary
		Cynops spp.	Skin, egg, ovary, muscle, blood
		Triturus spp.	Skin, egg, ovary, muscle, blood
		Ambystoma sp.	Skin, egg, ovary, muscle
		Paramesotriton sp.	Skin, egg, ovary, muscle
		Polypedates sp.	Skin
		Atelopus spp.	Skin
		Colostethus spp.	Skin
9	Red Clacareous alga	Jania spp.	Whole body
10	Dinoflagellate	*Alexandrium tamarense*	Whole bodv

12 Appendix 7 Tetrodotoxin summary – Leo Schep

Routes of Exposure

Tetrodotoxin is absorbed from the alimentary canal, respiratory tract and through intact skin.

Tetrodotoxin poisoning most often occurs via the ingestion of tetraodontiform fish. Tetrodotoxin can also be absorbed through skin, a case has been reported where the victim had been dissecting a large puffer for 2 days and developed general malaise, intense headache, gastrointestinal complaints, and itching and a rash on the face and hands.[2] Dermal absorption would only be significant if the exposure was for extended periods of time.

Onset/Duration of Symptoms

Poisoning may be rapid in onset with the first symptoms appearing within 5 to 15 minutes of ingestion, (death has been reported within 17 minutes),[3] however symptoms usually develop within 10 to 45 minutes of ingestion[4,5] and most patients will have their symptoms develop within 6 hours,[6] rarely symptoms may be delayed up to 18 hours post ingestion.[7]

The more rapid the onset of symptoms the more severe the degree of poisoning. With treatment most symptoms subside with complete recovery usually in 24 to 48 hours.[4,8,9,10]

Severity of Poisoning

Tetrodotoxin poisoning has been divided into 4 grades based on severity of poisoning.[11]

Grade 1
- Numbness around mouth
- Paresthesia
- Nausea

Grade 2
- Numbness of face, tongue and other areas
- Early motor paralysis and incoordination
- Slurred speech
- Reflexes normal

Grade 3
- Widespread paralysis
- Dyspnea
- Hypotension
- Fixed dilated pupils
- Patient still conscious

Grade 4
- Severe respiratory failure and hypoxia
- Hypotension
- Bradycardia
- Cardiac dysrhythmias

Patient may be unconscious
Death due to respiratory failure

References

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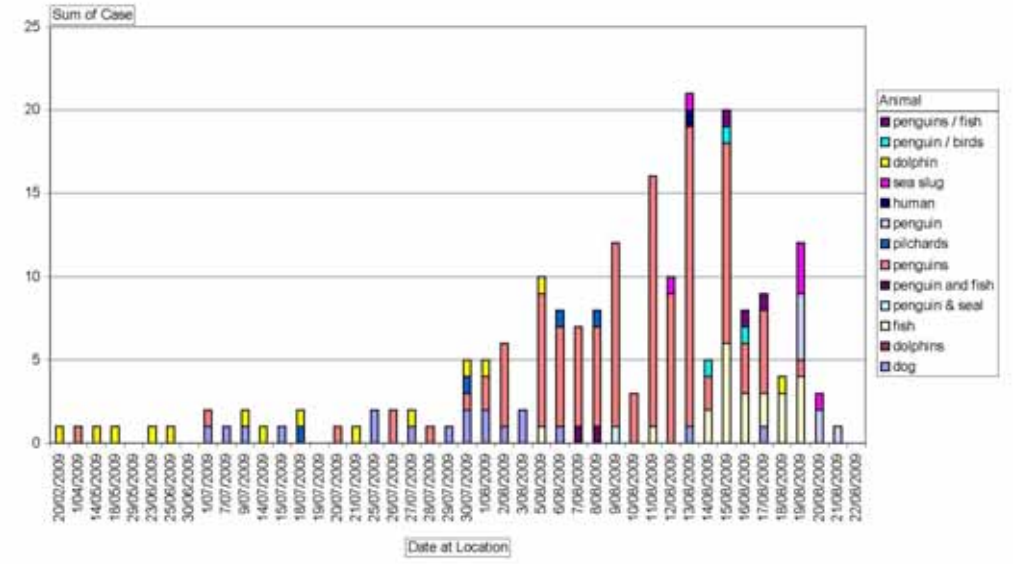
13 Appendix 8 Case Reports of Poisoned dogs

	Reported	Detail of call	Place	Onset	Fate	Clinical signs
1	5/08/09	dog sudden death	Cheltenham Beach	01/07/09	Died	Vomiting, bradycardia
2	6/08/09	dog sick after beach, recovered	Narrowneck Beach, Devonport	07/07/09	Recovered	Vomiting within 15 minutes of beach, recovered after vet visit
3	7/08/09	dog sick after beach, death	Narrowneck Beach, Devonport	09/07/09	Died	Vomiting within 20 minutes of beach, vet visit, died within an hour of beach
4	6/08/09	Dog got sick 15 July after being on Eastern Beach. vomited, diarrhoea and sleepy - picked up couple days later	Eastern Beach, Howich area	15/07/09	Recovered	Vomited, diarrhea and sleepy - recovery over 2 days
5	5/08/09	Dog euth after uncontrollable seizures	Cox's Beach	25/07/09	Euthanized	Neurological signs
6	6/08/09	dog sick after beach, recovered	Browns Bay / Waiake Beach	25/07/09	Recovered	Neurological signs, gait change within 15 minutes of beach, recovered gradually
7	5/08/09	Dog poisoned angry about cost	Kohimarama Beach	27/07/09	?	
8	5/08/09	dog swam at Karaka Bay 29/30July. Hosp next 4 days at Kohimarama vets with unexplained illness.	Karaka Bay	29/07/09	Recovered	Neurological signs
9	5/08/09	dog sick, saw vet	Torbay area, Long Beach	30/07/09	Recovered	Gagging, diarrhoea, agitated within 4hrs of beach visit.

10	6/08/09	dog developed CNS signs 36 hrs post swimming at Browns Bay. Walking in cicles, disorientated.	Browns Beach	30/07/09	Recovered	Circling, disorientated, distressed. Visited vet. No diagnosis given
11	6/08/09	Sick dog 1 hr after swimming is sea Clinical signs started at the beach	Waiomu Beach, Thames	01/08/09	Recovered	Vomiting within 1 hr, retching continuing for 36 hrs plus generally weak and shaky
12	6/08/09	Dog sick following walk near Browns Bay. Awaiting hx from vets.	Browns Bay	01/08/09	Recovered	Vomiting, hosp 3 days
13		dog stopped running on beach walk / swim Clinical signs started at the beach	Onetangi bay beach, Waiheke	02/08/09	Recovered	Neurological signs, cardiac signs and diarrhoea, gradual recovery 3ds
14		dog sick after beach, death Clinical signs started at the beach	Narrowneck Beach, Devonport	03/08/09	Died	Vomiting within 15 minutes of beach, death 1 and half hours after beach
15		dog sick after beach, recovered Clinical signs started at the beach	Narrowneck Beach, Devonport	03/08/09	Recovered	Vomiting within 10 minutes of beach, vet visit, likely to recover
16	6/08/09	dog lost near Tamaki estruary and found dead next morning Clinical signs unknown	Manor Park Road, Tamaki estuary	06/08/09	Died	Found dead
17	13/08/09	Vomiting, salivation, mild hindlimb ataxia, muscle tremors and fasiculations, hypothermic, bradyarrythmia Onset of signs 48 hrs after beaches?	Takapuna beach & Pt Chevalier	7/08/09	Recovered	Vomiting, salivation, ataxia, bradyarrythmia + hypothermic
18	22/08/09	Acute onset vomiting, disorientated and weak after walk Clinical signs started at the beach	Waiomu Beach, Thames	22/08/09	Recovered	Vomiting profusely and rapidly lethargic and ataxic after beach walk at 12pm. Was seen with a dead bird it picked up on beach.

14 Appendix 9 Reports to the MAF 0800 80 99 66 number



Situation Report
Animals affected on beaches, Auckland

Date Monday 24 August 2009

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12.4 Appendix D: A review of tetrodotoxins in various taxa

Fish

Tetrodotoxin is named from the family of puffer fishes (Tetraodontidae) that most famously contain high levels of this toxin and are most often associated with human poisoning as this fish is traditionally considered a delicacy in Japan, Korea and China. Records of puffer fish poisoning from China and Japan date back over 2000 years.

At least 22 different species of Asian marine and brackish-water puffer fish have been shown to contain high levels of TTX though seasonal, individual and spatial variations of toxicity and toxin composition have been observed (Miyazawa & Noguchi, 2001, Noguchi & Arakawa 2008). TTX in puffer-fish may take the form of several chemical analogues and may also co-occur with saxitoxins (STXs). The highest levels of TTX in puffer fish is found in the liver, ovaries, skin and intestine. The distribution of TTX in the tissues appears to some extent to be species specific and the toxicity of puffer fish can show remarkable individual and regional variations.

In marine puffers the liver generally has the highest toxicity except during spawning when TTX levels in the ovaries become high. TTX has been found in puffer-fish eggs and they excrete TTXs from the skin when threatened. TTX contaminated gobies have caused poisoning of humans and wildlife in Japan and Taiwan and these have reputably been used as natural rodenticide.

TTX is naturally found in the prey species of puffer-fish and fish (*Takifugu* rubripes) that are commonly toxic, when raised on TTX free diets in sea-cages and land based aquaria have been conclusively proven to not contain any TTX (Noguchi *et al.* 2006a). Conversely it has been shown (Noguchi *et al.* 2006b) that non toxic puffer fish can be made toxic by feeding with a TTX containing diet. This provides very strong evidence that wild puffer-fish become intoxicated through the food chain (presumably by preying on starfish, crustanceans, flatworms etc) and artificially raised fish are suitable for human consumption. Interestingly, non toxic puffer fish apparently retain their resistance to the effects of TTX (Saito *et al.* 1984) as shown by inoculation of fish with high doses of pure TTX. Matsumura (1998) showed that TTX production occurred in puffer fish embryos and increased continuously as the embryos developed.

Amphibians

Newts from Japan, China, USA and Italy have been shown to contain TTX with the skin and ovaries generally showing higher toxicity than muscle, liver, stomach intestine and liver. Very high levels of TTX (up to 3.3mg/g skin; Hanifin et al 2002) have been observed in the newt *Taricha granulsoa* and these have been shown to significantly increase in individual animals over a one year period in a captive environment.

TTX has been found in the skin and eggs of a number of species of frogs and toads from South and Central America (Pires *et al.* 2002; Pires *et al.* 2005). Some species have been shown to retain high levels of toxicity over long periods. The origin of TTX in amphibians is unknown, TTX producing bacteria have never been found to be associated with these animals and endogenous production has been suggested.

Arthropods

The eggs of horseshoe crabs (*Carcinoscorpius rotundicauda*) are regarded as a delicacy in Thailand and their contamination with TTX has resulted in serious outbreaks of food poisoning.

Horseshoe crab eggs show higher toxicity than other tissues such as testis and viscera and it is assumed that the accumulation of TTX in the eggs is a predatory defence mechanism.

TTX has been shown to be a major toxin in a range of species of xanthid crabs in Japan, the Philippines, Singapore and Taiwan (Saito *et al.* 2006, Noguchi *et al.* 1983). However, there appears to be no explanation as to where the toxin is acquired by the crabs.

Molluscs

Blue ringed octopus produce TTX within their salivary glands which gives them their fearsome reputation (Sheumack and Howden, 1978). The direct introduction of TTX into the blood stream via the octopus bite can cause the death of an adult human within 20 minutes if not treated immediately. More than 16 species of gastropod have been shown to contain TTX and consumption of these animals has caused numerous human illnesses, including fatalities (Hwang *et al.* 1990). Most gastropods shown to contain TTX are predators and scavengers (Table 1). In Japan there has been a belief that gastropods may become toxic by feeding on toxic viscera of puffer-fish that have been discarded by fishermen (Yasumtoto *et al.* 1981) or have died after spawning (Noguchi and Arakawa 2008).

Echinoderms

TTXs have been identified in at least four species of starfish from Japan and Taiwan. TTX concentrations up to 16,800 MU/specimen have been observed in the starfish *Astropecten scoparius* (Lin and Hwang, 2001) with highest levels in the gonads and viscera. There are wide seasonal and individual variations in toxicity and the gonads increase in toxicity as the animals reach sexual maturity. *Astropecten. scoparius* switch seasonally between preying on gastropods (snails) and bivalves. It was only when preying on the gastropods (which themselves contained TTX) that the levels in the starfish increase.

Further food chain transmission of TTX has been observed in the accumulation of TTX in the large predatory Trumpet shell (*Charonia sauliae*) preying on the starfish *A. polyacanthus* (Noguchi *et al.* 1982). The transfer and accumulation of toxin via feeding of starfish on small gastropods then the feeding of large gastropods on the starfish provides good evidence for food chain transmission of TTX.

Worms

Miyazawa *et al.* (1986) first reported on the occurrence of TTX in marine flat worm (*Planocera multitentaculata*). A subsequent study of the anatomical distribution of toxins (Miyazawa *et al.* 1987) showed the oviducts had the highest toxicity of any tissue and the eggs laid by these flatworms were extremely toxic (up to 10,700 MU/g). Presumably the TTX plays an anti-predator role in the protection of the eggs.

The Japanese ribbon worm *Cephalothrix linearis* has been shown (Ali *et al.* 1990) to have very high levels of TTX in its tissues (up to 13,000 MU/g whole body). The toxin was especially concentrated within the proboscis of this predatory worm and mucous secretions also contained high levels of TTX. Because these worms have the ability to secrete considerable amounts of toxin when stimulated, TTX may play both defensive and offensive roles in this organism. Research on the chemistry of *C. linearis* toxins (Noguchi *et al.* 1991) has shown that high potency TTX may be produced in the animal from a low toxicity precursor tetrodonic acid-like substance.

Planktonic arrow-worms and flatworms have been shown to use TTX to paralyze mobile prey (Thuesen *et al.* 1988; Ritson-Williams *et al.* 2006). TTX has also been detected in nematode

and annelid worms (Kogure *et al.* 1996) and it has been suggested that these play an important role in the accumulation and transfer of TTX in the environment.

Algae

As far as we are aware there is only one account of TTX being associated with algae. This was with the red tropical calcareous algae *Jania* sp. (Yasumoto *et al.* 1989) but the TTX content of the algae was so variable that it was presumed to have been derived from symbiotic or epiphytic bacteria, and in fact TTX producing bacteria were isolated from it. It seems likely that if heterotrophic bacteria are the major source of TTX in the environment, then they are likely to be associated with a photosynthetic organism from which they could obtain organic material for their sustenance. It may be significant that a red branched calcareous alga was found within the gut contents of *Pleurobranchea maculata* from the Hauraki Gulf beaches.

As yet there are no accounts of cyanobacteria producing TTX however this is a possibility that cannot be ignored as various cyanobacteria species are well known to synthesize similar compounds such as saxitoxins (*e.g.* Kellmann *et al.* 2008).

Bacteria as the source of TTX

TTX and anhydroTTX were detected in *Vibrios* isolated from the intestines of the xanthid crab *A. floridus* (Noguchi *et al.* 1986). Yasumoto *et al.* (1986) also isolated two bacteria (*Shewanella alga* and *Alteromonas tetraodonis*) from the red calcareous alga *Jania* sp. The bacteria were cultured and TTX detected in the culture broth.

Bacteria were isolated from the starfish *A. polyacanthu*s and puffer fish *Fugu vermicularis* and shown to produce TTX (Noguchi *et al.* 1987). All strains of *Vibrio alginolyticus* produced TTX.

Analyses by Carroll *et al.* (2003) strongly suggested a relationship between *Vibrio* bacteria and TTX-like chemicals in nemertean worms and they suggested that the toxins were utilized as a chemical defense against predators.

TTX may be produced by marine and freshwater bacteria that are common inhabitants of sediments. Kogure *et al.* (1988) showed TTX production by bacteria isolated from deep (4000 m) offshore sediments and Do *et al.* (1993) demonstrated the presence of TTX in freshwater lake sediments. Hamasaki *et al.* (1994) also showed that material caught in sediment traps in a coastal inlet contained TTX and related substances. They suggested that sinking particles are one of the sources of TTX in the marine environment and may play a role in the contamination of marine organisms.

Controversially, has been suggested that TTXs putatively produced in bacterial cultures are actually artifacts generated by components of the culture medium (Matsumura, 1995), but this has been strongly refuted by others.

A study by Gallacher and Birkbeck (1993) showed that TTX production by *Aletromonas tetraodonis* occurred during stationary phase and was up to 100 fold higher in phosphate limited cultures.

A TTX producing actinomycete (*Nocardiopsis dassonville)* has been isolated from the ovaries of the puffer fish (Wu *et al.* 2005) although it is not proven that this bacterium is actually responsible for the TTX found in the animals tissues.

Evidence for the endogenous origin of TTX

Several species of newts, frogs and toads carry high concentrations of TTX in their skin as a protection against predation. A number of researchers have come to the conclusion that it is

unlikely that endosymbiotic bacteria are the origin of TTX in toxic newts (Lehman *et al.* 2004) because newts have egg-yolks, embryos and newly hatched larvae containing TTX the origin of which is presumably under hormonal control by the mother. Also no TTX producing bacteria have been isolated from any amphibian species which posses TTX to date (Pires *et al.* 2005).

Specialized structures (granular glands) exist in the skin of newts associated with the storage and secretion of TTX. These glands do not contain bacteria (Lehman *et al.* 2004). Cardall *et al.* 2004 experimentally induced newts to release TTX from their skin by electrical stimulation and found that after 9 months in a captive environment they had significantly regenerated the levels of TTX in their skin. Other studies have demonstrated correlations between the density of granular glands in the skin of newts and the levels of TTX (Tsuruda *et al.* 2002; Hanifin *et al.* 2004); these data were consistent with the hypothesis that newts produce their own TTX.

TTX resistance

TTX exerts it effect by having a very high affinity for a receptor on the sodium channels in the membranes of motor neurons of vertebrates and invertebrates. Attachment of TTX to the sodium channel receptor blocks the transmission of nerve impulses, resulting in muscular paralysis. Saxitoxins (STXs) produced by planktonic dinoflagellates and cyanobacteria have the same effect. Recent research has shown that minor mutations in the genes that code for a key protein in the sodium channel receptor can make animals immune to the effect of the toxins. These mutations are selected for in populations of animals (*e.g.* soft shell clams, garter snakes, puffer-fish) that are habitually exposed to these toxins. As a consequence populations can accumulate very high levels of toxin that would be fatal to non immune animals in other populations of the same species.

There are two critical amino acid residues in the sodium channel receptors of puffer fish that confer TTX resistance (Murata *et al.* 2008). Garter snake populations acquire resistance to TTX in the skin of toxic newts via a single point mutation in sodium channel receptors (Geffeney *et al.* 2005).Soft shell clam (*Mya arenaria*) populations acquire STX resistance via selection of a point mutation resulting in a single amino acid change in the sodium channel receptor, resulting in clams accumulating much higher levels of STX than non resistant populations (Bricelj *et al.* 2005)

In addition to this, specific proteins that bind to TTX have been found in the in the body fluids of crabs and puffer–fish. These may provide a mechanism for the storage and transport of high levels of toxin in these animals. The crab *Hemigrapsus sanguineus* is highly resistant to TTX, due to a high molecular weight TTX binding protein in body fluids (Shiomi *et al.* 1992.)

A TTX binding protein that may play a role in toxin accumulation has also been isolated from the blood plasma of the puffer-fish *Takifugu niphobles* (Matsui *et al.* 2000). This is possible explanation why puffers and not other fish species sequester TTX when fed a TTX containing diet.

Although it is speculative, a recently acquired resistance to TTX, due to an increase in the natural levels of this toxin in its habitat, may have resulted in the extremely high levels of TTX observed in *P. maculata* collected from the area near Narrow Neck beach.

Table 1 Gastropod molluscs previously implicated in tetrodotoxin contamination

Species	Common name/diet	Notes	Reference
Babylonia japonica	Japanese Ivory Shell	TTX detected + human poisoning	Yasumoto *et al.* 1981[1] Noguchi *et al.* 1981
Charonia sauliae	Triton Trumpet Shell	TTX detected	Narita *et al.* 1981
Natica lineate *N. lineata*	Moon Snails Predators feeding on bivalves and other snails	TTX detected + human poisoning	Shiu *et al.* 2003
Niotha clathrata	Dog Welks/Nassa mud snails mud snails soft shore scavengers	TTX detected + human poisoning	Hwang *et al.* 2002[2] Jeon *et al.* 1984 Cheng *et al.* 1995[3]
Nassarius semiplicatus *N. glans*	Dog Welks/Nassa mud snails soft shore scavengers	TTX detected + fatal human poisoning	Wang *et al.* 2008[4] Yin *et al.* 2005[5]
Oliva miniacea *O. mustelina* *O. nirasei*	Olive Shell Scavengers and predator on bivalves	TTX detected + fatal human poisoning	Hwang *et al.* 2003 Spencer *et al.* 1986
Polinices didyma	Moon Snail. Predators feeding on bivalves and other snails	TTX detected + human poisoning	Shiu *et al.* 2003.
Rapana rapiformis *R. venosa venosa*	Murex/Rock shells Scavengers and predators on bivalves	TTX detected	Hwang *et al.* 1991
Tutufa lissostoma	Frog Shell Predators of echinoderms	TTX detected	Noguchi *et al.* 1984
Zeuxis sufflatus *Z. siquijorensis*	Dog Welks/ Nassa mud snails mud snails soft shore scavengers	TTX detected + human poisoning	Hwang *et al.* 2002[6]

1. TTX contaminated specimens of *B. japonica* were only found at one out of five locations over ~ 20km of coastline in Wakasa Bay, Japan.
2. .Highest TTX in spring and autumn, maximum toxicity = 1,900 MU/?g specimen, TTX secreted in response to electric shock.
3. No relationship found between toxicity of shellfish and TTX producing bacteria in tissues
4. TTX producing bacteria (mainly *Vibrios*) isolated from *N. semiplicatus* but toxin production low.
5. Maximum levels of TTX in *Nassauris glans* of 10,361 MU/g
6. Maximum toxicity = 1,640 MU/ specimen, TTX concentrated in digestive gland

12.5 Appendix E: Cawthron Report No.1652

Report On Cawthron's Investigations Into Dog Deaths On Auckland's East Coast Beaches

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Prepared for

Auckland Regional Council
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1. INTRODUCTION

The Cawthron Institute was asked to investigate the cause of deaths of dogs on beaches located on Auckland's east coast adjacent to the Hauraki Gulf in August 2009. Initially tests were conducted on dog vomit from three animals that had died after consuming an unknown material. The dogs were reported to experience either a sudden onset of repeated vomiting at the beach followed by death within hours or if the dog survived, a rapid recovery or the onset of neurological symptoms including seizures, unusual behaviour and altered mental status within hours of being at the beach and gradual recovery or deterioration.

Analysis of the dog vomit samples revealed no algal material and, in response to further incidents of sick dogs and reports of dead penguins and pilchards, two Cawthron researchers travelled to Auckland on Friday August 7th 2009. The scientists visited Narrowneck, Cheltenham and Tamaki beaches and collected 13 samples including sea water, algal material, sponges, seaweed, and molluscs. They also deployed SPATT bags, which involves suspending in the water body small bags containing adsorption substrates which accumulate toxins, thus providing a method of assessing extra-cellular toxins over an extended period of time. In addition to these samples, water and biota samples were sent to Cawthron by the Department of Conservation and North Shore City.

This work was funded by the Cawthron Institute under the Seafood Safety programme (CAWX0307) and resulted in the identification of a toxic sea slug and the identification of the potent neurotoxin tetrodotoxin (TTX) in the sea slug.

Further testing was then commissioned by Auckland Regional Council (ARC) to confirm the presence of TTX, and to test more samples for TTX.

2. SAMPLES

Samples received by Cawthron were as follows:

Table 1. Samples sent to Cawthron

Sample Type	Sender	Label	Date Sampled	Date Received
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit AU09-14878	10 Jul 2009	14 Jul 2009
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit #1 AU09-16842	4 Aug 2009	5 Aug 2009
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit #2 AU09-16842	4 Aug 2009	5 Aug 2009

Sample Type	Sender	Label	Date Sampled	Date Received
Dog vomit	Gribbles Veterinary, Auckland	Dog vomit #3 AU09-16758	3 Aug 2009	5 Aug 2009
Jelly fish	Ciaran Edwards - North Shore City		3 Aug 2009	7 Aug 2009
Dried seaweed – Narrowneck Beach	Ciaran Edwards - North Shore City		3 Aug 2009	4 Aug 2009
Damp seaweed – many types from Narrowneck Beach	Bill Trusewich - Department of Conservation		4 Aug 2009	4 Aug 2009
Dead pilchards from Orewa Beach	Rodney DC	V637	Unknown, mid Jul	11 Aug 2009
Sea slug from Cheltenham Beach	Bill Trusewich - DoC	V639	12 Aug 2009	13 Aug 2009
Sea slug from Narrow Neck	Marcus Cameron ARC – EtOH preserved	V640	12 Aug 2009	13 Aug 2009
Sea slug from Narrow Neck	Marcus Cameron - ARC	V641	12 Aug 2009	13 Aug 2009
Dog vomit	Glenfield Vets	V642	7 Aug 2009	18 Aug 2009
Seawater – from bag containing V644	Peter Williams - ARC	V643	17 Aug 2009	18 Aug 2009
Sea slugs from Cheltenham Beach	Peter Williams - ARC	V644	17 Aug 2009	18 Aug 2009
Sea slugs from Narrow Neck Beach	Peter Williams - ARC	V645	17 Aug 2009	18 Aug 2009
Sea slugs from Rocks Road, Nelson	Rod Asher - Cawthron	V646	18 Aug 2009	18 Aug 2009
Pufferfish (Long Bay)	Rosemary Barraclough – Massey Albany	V647 – V650	14 Aug 2009	19 Aug 2009
Oysters (Brown's Bay)	Ciaran Edwards - North Shore City	V651	12 Aug 2009	20 Aug 2009
Oysters (Long Bay)	Peter Williams - ARC	V652	19 Aug 2009	20 Aug 2009
Pilchards (Long Bay)	Rosemary Barraclough – Massey Albany	V654	28 Jul 2009	20 Aug 2009
Sea Cucumber	ARC	V655	Not Known	21 Aug 2009
Intestine and Liver from dead dog	Dog died on Stanmore Bay	V656 & V657		21 Aug 2009
Sea slugs from Sth Narrow Neck Beach	Peter Williams - ARC	V660	21 Aug 2009	22 Aug 2009
Sea slugs from between Cheltenham and Narrow Neck Beach	Peter Williams - ARC	V661	21 Aug 2009	22 Aug 2009

Sample Type	Sender	Label	Date Sampled	Date Received
Sea slugs from mid Narrow Neck Beach	Peter Williams - ARC	V662	21 Aug 2009	22 Aug 2009
Sea slugs from Nth Narrow Neck Beach	Peter Williams - ARC	V663	21 Aug 2009	22 Aug 2009

Table 2 **Samples collected by Cawthron on the 7 August 2009**

Sample Type	Sampling Location	Date Sampled	Date Received
Sediment/sand	Sand surface Cheltenham Beach	7 Aug 2009	10 Aug 2009
Seawater	Tamaki	7 Aug 2009	10 Aug 2009
Seawater	Narrowneck Beach, freshwater inlet	7 Aug 2009	10 Aug 2009
Seawater	Narrowneck Beach, freshwater inlet	7 Aug 2009	10 Aug 2009
Scraping (green)	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Limpets	Narrowneck Beach (northern end) - V659	7 Aug 2009	10 Aug 2009
Mussel scraping	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Seaweed and sponge from high tide line	Narrowneck Beach (northern end)	7 Aug 2009	10 Aug 2009
Greenshell mussels	Cheltenham Beach (southern end) – V638	7 Aug 2009	10 Aug 2009
Algal mat	Cheltenham Beach (southern end)	7 Aug 2009	10 Aug 2009
Sponge low tide	Cheltenham Beach (southern end)	7 Aug 2009	10 Aug 2009
Three sea slugs (50-90mm)	Narrowneck Beach	7 Aug 2009	10 Aug 2009
Small black unidentified slugs (10-15 mm)	Narrowneck Beach (northern end) – V658	7 Aug 2009	10 Aug 2009

3. METHODS AND RESULTS

3.1. Algal, toxicological and common marine toxin analysis

3.1.1. Microscopic Analysis

Sub-samples of the dog vomit were taken and approximately 5 mL were used for species identification and enumeration using an inverted Olympus microscope (CKX41) and Utermöhl settling chambers (Utermöhl 1958). Seaweed samples were washed in freshwater and three 10 mL sub-samples were taken for species identification as described above. Seaweed samples were also checked for the presence of epiphytic species.

Watercare Laboratory Services Ltd collected water samples off 28 North Shore beaches on 6th August, 18 of the samples were preserved with Lugols. The samples were received at Cawthron 7th August and the 18 preserved samples were analysed by light microscopy for microalgae species identification and enumeration. The biomass measured across all 18 samples was low, this is normal during the winter months and consistent with results found in the routine biotoxin monitoring programme. There were some toxic species identified, however numbers were well below the trigger levels set in the Animal Products - Specifications for Bivalve Molluscan Shellfish Notice 2006 (NZFSA).

Table 3 **Results of microscopic analysis of samples**

Sample type and location	Algae/cyanobacteria identified in sample
Dog vomit AU09-14878	No algal/cyanobacterial material observed
Dog vomit #1 AU09-16842	Small diatoms present
Dog vomit #2 AU09-16842	No algal/cyanobacterial material observed
Dog vomit AU09-16758	No algal/cyanobacterial material observed
Dried seaweed – Narrowneck Beach	No potentially toxic cyanobacteria/algae observed. A mixture of algal species present all in low abundance
Damp seaweed – many types from Narrowneck Beach	No potentially toxic cyanobacteria/algae observed. A mixture of non-toxic algal species present all in low abundance.
Algal mat, Cheltenham Beach+	Sheathed diatoms (*Naviculoid* spp.)
Sediment/sand, Cheltenham Beach+	Mixed diatoms and *Prorocentrum* cf. *triestinum**, *P. maculosum* and *Gymnodinium sp.*
Seawater, Tamaki+	Debris
Seawater, Narrowneck Beach freshwater inlet+	Debris
Seawater, Narrowneck Beach freshwater inlet+	Debris
Scraping, Narrowneck Beach green+	Cyanobacterial mat - *Oscillatoriales (Phormidium* –like)

+ samples collected by Cawthron staff.

* Cells or filaments of these species were isolated for future culturing and identification.

3.1.2. Identification of samples

Sea slugs collected from Narrowneck and Cheltenham beaches were identified as *Pleurobranchea maculata*. The small black slugs (V658) collected from rocks at northern end of Narrowneck beach were identified as *Onchidella nigricans*. Puffer fish (V647 & V648) were identified as Porcupine fish *Tragulichthys* (formerly *Allomycterus*) *jaculiferus*. Limpets (V659) collected from rocks at northern end of Narrowneck beach were identified as *Celiana radians*. Sea cucumber (V655) was identified as *Stichopus sp.*

3.1.3. Sample preparation for toxicological screening

1. A sub-sample (32.6 g) of dog vomit AU09-16842 was extracted three times with dichloromethane (DCM), The DCM extract was dried down and transferred to a 20 mL vial with methanol. Methanol was evaporated to dryness under nitrogen.
2. The remaining aqueous fraction from the DCM extraction (above) was boiled for 5 min, dried by rotary evaporation. The dried residue was extracted with methanol and transferred to a 20 mL vial with methanol and evaporated to dryness under nitrogen

3. A sub-sample (50 g) of dog vomit on sand Ref # 7175313 was extracted with methanol and dried by rotary evaporation. The residue was transferred to a 20 mL vial and dried under nitrogen.
4. A sub-sample of the jellyfish was lyophilized (freeze-dried). A sub-sample (10 g) of lyophilized material was extracted with methanol and dried in a 20 mL vial under nitrogen.
5. A sub-sample (20 g) of dried seaweed collected from Narrowneck Beach by Ciaran Edwards on the 03 Aug 2009 was extracted with methanol. The extract was transferred to a 20 mL vial and dried down under nitrogen.
6. A sub-sample of the sponge (10 g) collected from Cheltenham Beach on 7 August 2009 was extracted with methanol and the extract was dried in a 20 mL vial under nitrogen.
7. Damp seaweed collected from Narrowneck Beach by Bill Trusewich on 4 August 2009 was soaked in freshwater overnight. The water was a brown colour and contained a range of micro-algae in low abundance. A 500 mL sub-sample of water was dried by rotary evaporation. The resulting residue was dissolved in methanol, transferred to a 20 mL vial and the methanol removed under nitrogen.
8. Three sea slugs collected from Narrowneck Beach on 7 August 2009 were homogenized. A sub-sample (20 g) of homogenate was extracted with methanol. The methanol was removed by rotary evaporation, the resulting residue was transferred with methanol to a 20 mL vial and the methanol was removed under nitrogen.
9. Greenshell mussels collected from Cheltenham Beach on 7 August 2009 were shucked and homogenized. A sub-sample (20 g) of mussel homogenate was extracted with methanol. The methanol was removed by rotary evaporation. The resulting residue was transferred with methanol to a 20 mL vial and methanol was removed under nitrogen.
10. A sub-sample (10 g) of algal mat (*Naviculoid* spp.) collected from Cheltenham Beach on 7 August 2009 was extracted with methanol. The methanol was removed by rotary evaporation. The resulting residue was transferred with methanol to a 20 mL vial and methanol was removed under nitrogen.

3.1.4. Toxicology of methanol extracts.

Samples were taken up in 1% Tween 60 in saline. Aliquots of the resulting solutions or suspensions were diluted to 1 ml in Tween-saline and injected intraperitoneally into Swiss albino mice, of body weight between 18 and 22 g. Although several samples affected the mice only one sample – sea slugs – exhibited significant toxicity. Refer to Supplement 1 for more information.

3.1.5. Toxin analysis

Anatoxin analysis

A *Phormidium*-like species was detected in an algal sample collected from Narrowneck Beach. Freshwater species from this genera are known to produce the neurotoxins anatoxin-a and homoanatoxin-a (Wood et al. 2007). This sample was analysed for anatoxin-a and homo-anatoxin by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in Wood et al. 2007. No anatoxins were detected in this analysis (Limit of detection (LOD), 0.2 µg/L). A sub-sample of sea slug homogenate (2 g) was extracted with 18 mL of 90% methanol

and the resulting extract analysed for anatoxins as described above. No anatoxins were detected in this sample (<10 µg/kg).

Lipophilic marine biotoxin analysis

A 2g sub-sample of sea slug and oysters from Long Bay (V652) were homogenized and extracted with 18 mL of 90% methanol. The methanolic extracts were analysed for 26 marine biotoxins by LC-MS/MS (McNabb 2005). No toxins were detected in either sample, results and LOD's are given in Table 4.

Table 4 LC-MS analysis for lipophilic toxins, ND = not detected.

Toxin	Limit of detection (mg/kg)	Concentration of toxin detected (mg/kg)
Domoic acid	0.01	ND
Gymnodimine	0.05	ND
13-desmethyl-spirolide C	0.01	ND
13-desmethyl-spirolide D	0.01	ND
Pinnatoxin A	0.01	ND
Pinnatoxin D	0.01	ND
Pinnatoxin E	0.01	ND
Pinnatoxin F	0.01	ND
Pinnatoxin G	0.01	ND
Brevetoxin-B2	0.04	ND
S-desoxy-brevetoxin-B2	0.04	ND
Azaspiracid 1	0.01	ND
Azaspiracid 2	0.01	ND
Azaspiracid 3	0.01	ND
Pectenotoxin 1	0.01	ND
Pectenotoxin 2	0.01	ND
Pectenotoxin 6	0.01	ND
Pectenotoxin 11	0.01	ND
Pectenotoxin 2 seco acid	0.01	ND
Okadaic acid	0.01	ND
Dinophysis toxin 1	0.01	ND
Dinophysis toxin 2	0.01	ND
Yessotoxin	0.02	ND
45-hydroxy-yessotoxin	0.02	ND
Homoyessotoxin	0.02	ND
45-hydroxy-homoyessotoxin	0.02	ND

Saxitoxin analysis

The presence of saxitoxins was assessed in the sea slug sample and oysters (V652) using the Jellett Rapid PSP Test Kit (Jellett et al. 2002) according to the protocol supplied by the manufacturer. No toxins were detected.

Brevetoxin analysis

The presence of brevetoxins-2 and brevetoxins-3 was assessed in the sea slug sample using the methanol extract (see lipophilic toxin testing) using LC-MS (Cawthron method 40.106). No toxins were detected.

3.1.6. SPATT bags

The synthetic adsorbent, DIAION HP20 (Mitsubishi Chemical Corporation) was added to mesh bags as described in MacKenzie et al. (2004). SPATT bags were deployed off Narrowneck beach. These were not tested, however, due to the findings below (section 3.2) which identified a highly polar toxin that would not have adsorbed to the HP20 resin.

3.2. Testing for uncommon marine toxins

3.2.1. Palytoxin haemolysis testing

The presence of haemolytic components was assessed in the methanol extract of the sea slug sample using the Bignami haemolysis assay. No haemolytic activity was recorded in the dried material diluted to 20 mg/mL. The limit of detection for the assay was 1 pg of palytoxin equivalents/mL.

3.2.2. Liquid chromatography-mass spectrometry (LC-MS)

Full scan mass spectra were collected for the methanolic extract of toxic sea slugs using reverse phase LC elution and positive electrospray ionisation .A polar component in high concentration consistent with tetrodotoxin (TTX) was observed near the solvent front. Further investigation to confirm TTX in the sample was completed and a new LC-MS method was established to quantitatively determine TTX in extracts.

3.3. Tetrodotoxin testing

A sample of TTX from Acros Organics (CAS 4368-28-9, citrate free, 1mg) was dissolved in 10mL of 10% acetonitrile containing 0.1% formic acid. This solution was used for quantitative LC-MS analysis after recalibration using the mouse bioassay, which showed that the vial contained only approximately 0.25mg TTX, not 1mg as stated. All quantitative data in this report is based on this single TTX standard and therefore have significant uncertainty when comparing to other reported concentrations, however the relative amounts are accurate. A new standard of TTX has been ordered which will confirm absolute concentrations.

An LC-MS method was set up utilising a Waters Acquity™ UPLC and Waters Premier™ triple quadrupole mass spectrometer with electrospray source. A TosoHaas (Japan) TSK-GEL amide 80, 5µ, 2.0 × 250 mm column was used for LC separation with an acetonitrile/water gradient elution. The system was buffered with formic acid 50 mM and ammonium formate 2 mM. Mass channels monitored were: 320.1>162.1, 320.1>60.0 as optimised by infusion of pure TTX and fragmentation using argon. Additional mass channels were included for known TTX analogues: 318.1>162.1 (11-oxo TTX), 304.1>162.1 (11-deoxy TTX), 302.1>162.1 (anhydro TTX), 290.1>162.1 (11-nor TTX). Samples were extracted with 90% methanol, 2g plus 18mL (McNabb et al 2005) and the centrifuged extract analysed directly by the above LC-MS method.

Figure 1 shows LC-MS chromatograms proving the presence of TTX in sea slug and dog vomit samples. The main peak observed in all traces corresponds in LC retention time and MS characteristics to TTX. Low levels of 11-nor TTX were also found in sea slug and dog vomit samples.

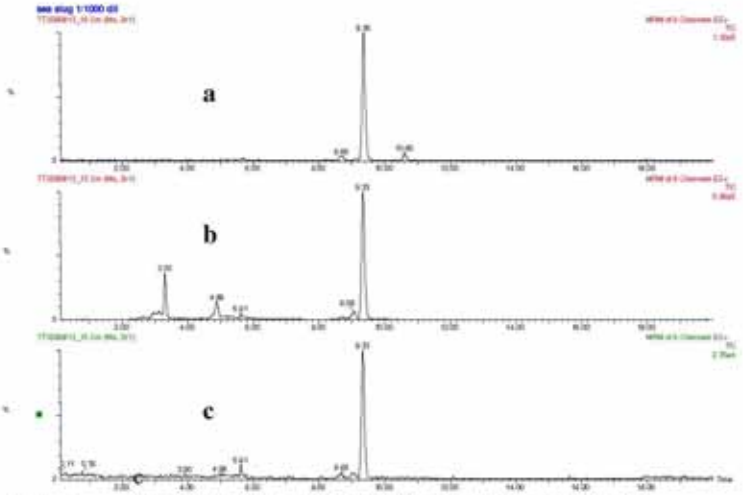

Figure 1 **LC-MS chromatogram a) pure TTX, b)dog vomit and c) sea slug.**

Figures 2 and 3 show chromatograms and daughter ion spectra collected using the LC method described but scanning for all the product ions produced from the 320.1 parent ion. This result confirms that TTX is present in the sea slug sample.

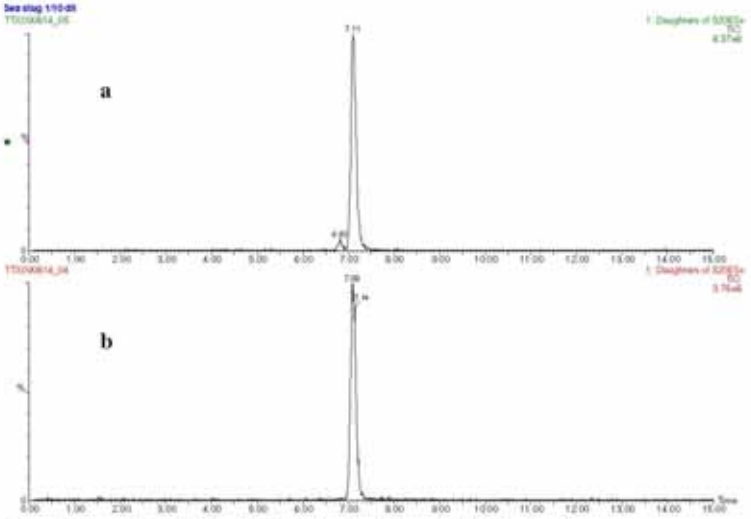

Figure 2 Daughter ion TIC. a) Sea slug, b) pure TTX standard.

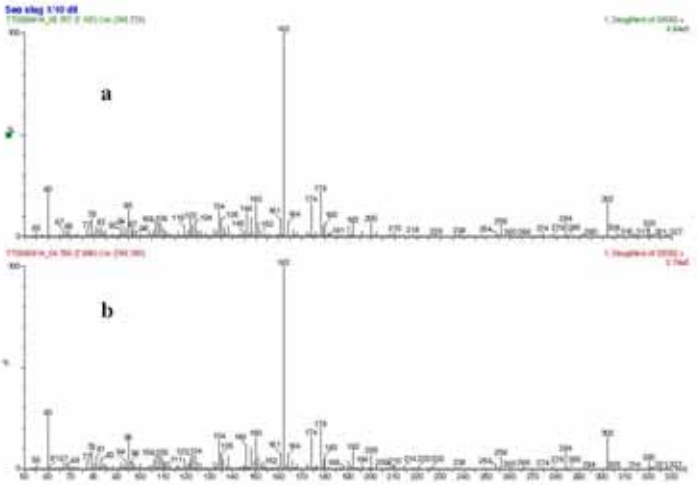

Figure 3 Daughter spectra fro fragmentation of MH$^+$ 320.1 . a) Sea slug, b) pure TTX standard.