SAXITOXIN BINDING PROTEINS: BIOLOGICAL PERSPECTIVES

Thesis submitted by

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BSc (Hons)

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For the degree of
Doctor of Philosophy

In the School of Pharmacy and Molecular Sciences
JAMES COOK UNIVERSITY
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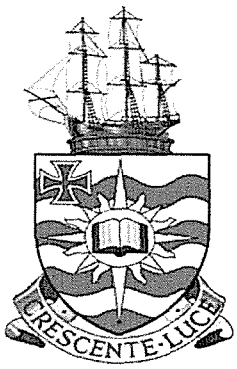

In collaboration with:
THE AUSTRALIAN INSTUTUTE OF MARINE SCIENCE
Marine Biotechnology
Townsville, Queensland, Australia.

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001).

All components of the project and reporting procedures were in compliance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, and the *Queensland Animal Care and Protection Act* (2001). The research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A745-02).

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STATEMENT ON THE CONTRIBUTION OF OTHERS

Financial

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- An Australian Postgraduate Award (APA), granted by James Cook University (JCU) and financed by the federal Department of Science, Education and Training (DEST), provided a generous stipend and was greatly appreciated.

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- The Australian Proteome Analysis Facility (APAF) performed tryptic digests of purified saxitoxin binding protein and subsequent peptide sequencing by manual QTOF mass spectrometry, on a fee for service basis.

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All other personal and scientific contributions are listed as acknowledgements for each relevant thesis chapter.

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PUBLICATIONS ARISING FROM THESIS

Each research chapter of this thesis will stand alone as at least one peer reviewed publication as detailed below. Additional publications and conference presentations indirectly relating to my thesis research are detailed as additional outcomes in Chapter 6.

Thesis Research Papers

- **Robertson, A.**, Stirling, D., Robillot, C., Llewellyn, L., Negri, A.P. (2004) First report of saxitoxin in octopi. *Toxicon*, **44**, 765- 771.

- **Robertson, A.**, Llewellyn, L. E., Negri, A. P. (2005) Development of a robust radio-receptor assay for discovery and screening of novel saxitoxin binding proteins and paralytic shellfish toxins. *Target Journal*: Environmental Science & Technology (in preparation).

- **Robertson, A.**, Negri, A.P., Llewellyn, L.E. (2005) Extending the phylogenetic distribution of saxitoxin binding proteins in search of functionality. Target Journal: *Toxicon* (in preparation).

- **Robertson, A.**, Llewellyn, L.E., Negri, A.P. (2005) Survey of a tropical saxitoxin "hotspot": Port Hedland, Western Australia. Target Journal: *Marine Biology* (in preparation).

- **Robertson, A.**, Swan, J., Negri, A. P., Burnell, J., Llewellyn, L., Wilson, K. (2005) Purification and partial sequencing of a saxitoxin binding protein from *Bufo marinus* plasma. Target Journal: *Biochemistry* (in preparation).

- **Robertson, A.**, Motti, C.A., Negri, A. P., Llewellyn, L.E. (2004) Evidence for endogenous ligands of saxiphilin in *Bufo marinus.* Target Journal: *Analytical Chemistry* (in preparation).

Thesis Conference Abstracts

Robertson, A. (2002) Paralytic shellfish toxin "hotspots" The Port Hedland Story. Oral presentation at 4*th Workshop of the Australian Research Network for Algal Toxins (ARNAT)*, Townsville, July 2002. (PhD research).

Robertson, A. and Llewellyn, L. E. (2002) Saxitoxin Binding Proteins: A Unique Suite of Receptors. Poster presented at ComBio 2002, Sydney, October 2002. (PhD research).

ABSTRACT

Saxitoxin binding protein (STXBP) is a functional classification which describes all proteins capable of binding to the paralytic shellfish toxin (PST), saxitoxin (STX). Based on this functionality, this group includes the voltage gated sodium channels (VGSCs), pufferfish STX and tetrodotoxin (TTX) binding proteins (PSTBPs) and saxiphilin (SXPN) which was been isolated from the amphibian *Rana catesbeiana*. Various activities and relationships of bullfrog SXPN have been elucidated including the ability to inhibit papain, human cathepsin B and L and the substantial homology of the amino acid sequence to transferrins (TFs). However, the biological role of SXPN has not been thoroughly examined and remains a mystery. It is likely that a detoxification mechanism exists in animals exposed to PSTs, and may explain the defined STX binding activity of soluble STXBPs. Therefore, the main objective of this thesis was to examine various aspects of the biological relationship between STX and soluble STXBPs to determine whether these proteins provide a defensive arsenal against PST intoxication.

Preliminary studies indicated that current methods for the detection of STXBPs were problematic and time intensive therefore several radio-receptor assays were developed and trialled to identify a suitable primary screening regimen for the detection and characterisation of these proteins. Assays utilising anion and cation exhange methods, protein binding and traditional charcoal radio-receptor methods were compared to published formats. A receptor binding filtration assay incorporating protein binding membranes of mixed cellulose esters (MCE) proved to be a robust method for the sensitive and accurate detection of STXBPs. This assay was easily converted for use as a PST screening tool and was validated in subsequent chapters using *Bufo marinus* plasma which is readily obtainable source of STXBPs.

With the aid of this optimised assay method, the diversity of soluble STX-specific receptors was investigated to extend previous phylogenetic surveys and identify any commonality between species that contain STXBPs. More than 1000 extracts, representing over 200 different species from the marine, freshwater and terrestrial

environment were investigated, resulting in the discovery of eight novel STXBPs extending the known phylogenetic diversity of STXBPs to include species from Onychophora and Mollusca. Seven of these species were characterised as STX specific hydrophilic receptors based on their ability to exclusively bind STX. In addition, a STXBP likely to belong to the PSTBP group was identified in the toad fish, *T. pleurogramma.*

Further examination of species collected from a verified STX "HotSpot" resulted in the identification of one additional STXBP in the extracts from the crab, *Lophozozymus octodentatus*. The occurrence of PSTs and diversity STX sources and vectors at this site were examined by means of radioreceptor assays including the centipede SXPN assay, rat brain synaptosome assay, liquid chromatography-fluorescence detection with post column oxidation, in addition to confirmatory mass spectrometric analysis. This study confirmed 3 new sources of PSTs in benthic food web of Port Hedland including the macroalgae, *Sargassum sp. Jania sp.2* and *Jania sp.3*. However, the STX levels in these species did not explain the extreme levels of STX observed in some vectors. A number of new PST vectors were identified from the bivalves *Tridacna squamosa, Pinctada albina sugilata, Saccostrea glomerata, Malleus regula* and the first incidence of STX in the octopi, *Octopus (Abdopus) sp. 5,* was reported. The lack of widespread STXBPs in the intertidal STX "Hot Spot" did not conclusively support the toxin defence hypothesis as a likely biological role of STXBPs.

Finally, an animal model, *B. marinus*, was selected for an in depth analysis of STXBPs. STXBP levels in the toad were ubiquitous across all life stages and within all tissues with the exception of the venom glands, and reflected previous reports of saxiphilin distribution in the bullfrog *R.. catesbeiana.* Interestingly, a positive correlation was demonstrated between environmental temperature and levels of STX binding activity in toad plasma. The STXBP isolated from *B. marinus* plasma was successfully purified, revealing an estimated protein size of 93 kDa and 3 peptide sequences which facilitated degenerate PCR experiments. Cloned STXBP-specific fragments of cDNA from toad liver were then cloned and the corresponding translated amino acid sequences revealed homology to the C-

lobe of both saxiphilin from *R. catesbeiana* (also known to contain the STX binding site) and a variety of TFs.

The biological role of soluble STXBPs remains a mystery but substantial advances have been made in terms of the diversity, function and relationships of these proteins. The potential application of STXBPs uncovered during this research, in both medical and research applications of PSP treatment and detection is substantial and the wealth of data collected will promote several new directions of research in this area.

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ABBREVIATIONS

[^{3}H] STX	tritiated saxitoxin
AEX	anion exchange
ANGIS	Australian National Genomic Information Service
ANOVA	analysis of variance
APAF	Australian Proteome Analysis Facility
ATCC	American Type Culture Collection
BLAST	Basic local alignment tool
bp	base pair
cDNA	complementary DNA
CEX	cation exchange
CHAR	charcoal
dcGTX 2	decarbamoyl gonyautoxin 2
dcGTX 3	decarbamoyl gonyautoxin 3
DCM	dichloromethane
dcSTX	decarbamoyl saxitoxin
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EtBr	ethidium bromide
GC 1	*Guanidinium catenatum* toxin 1
GC 2	*Guanidinium catenatum* toxin 2
GC 3	*Guanidinium catenatum* toxin 3
GAF-AAC	Genetic Analysis Facility- Advanced Analytical Centre
GF/B	glass fibre type B filters
GF/C	glass fibre type C filters
GTX 1	gonyautoxin 1
GTX 2	gonyautoxin 2
GTX 3	gonyautoxin 3
GTX 4	gonyautoxin 4
GTX 5	gonyautoxin 5
GTX 6	gonyautoxin 6
HAB	harmful algal bloom
HIC	hydrophobic interaction chromatography
HPLC	high performance liquid chromatography
IC_{50}	50 % inhibition constant
ICA	inhibitor of carbonic anhydrase
IPTG	isopropyl-beta-D-thiogalactoside
K_d	dissociation constant
kDa	kilo dalton
K_i	inhibition constant
LC-FD	liquid chromatography- fluorescence detection
LC-MS	liquid chromatography- mass spectrometry
LF	lactoferrin
LSC	liquid scintillation counting
MCE	mixed cellulose esters
Mol%	percentage of moles
MS-MS	mass spectrometry- mass spectrometry

MTF	melanotransferrin
MW	molecular weight
neoSTX	neosaxitoxin
NSW	New South Wales
OT	ovotransferrin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylene imine
PH	phosphocellulose
pKa	ionisation constant
PSP	paralytic shellfish poison
PST	paralytic shellfish toxin
PSTBP1	puffer-fish saxitoxin and tetrodotoxin binding protein 1
PSTBP2	puffer-fish saxitoxin and tetrodotoxin binding protein 2
PVDF	polyvinylidene difluoride
QLD	Queensland
RACE	papid amplification of cDNA ends
RNA	ribonucleic acid
RT	room temperature
SBE	saturation binding experiments
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	standard error of the mean
ST	Serotransferrin
STX	saxitoxin
STXBP	saxitoxin binding protein
STXdiHCl	saxitoxin dihydrochloride
SXPN	saxiphilin
TAE	Tris acetate buffer
TF	transferrin
TTX	tetrodotoxin
VGSC	voltage gated sodium channel
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

Theoretical Background and Objectives

1.1 Introduction

Saxitoxin binding protein (STXBP) is a functional classification of all proteins capable of binding to saxitoxin (STX; Fig. 1.1A), a potent neurotoxin from the paralytic shellfish toxin (PST) family. This protein group includes the membrane bound voltage gated sodium channels (VGSCs), puffer-fish STX and tetrodotoxin (TTX; Fig. 1.1B) binding proteins (PSTBPs) and saxiphilin (SXPN). Despite this functional similarity, there is no significant protein sequence or structural homology between members of this unique suite of receptors (see Appendix 1). This thesis will focus on the investigation of soluble STX-specific STXBPs such as SXPN and the primary focal point will be the relationship between these mobile receptors and their only confirmed ligand, STX.

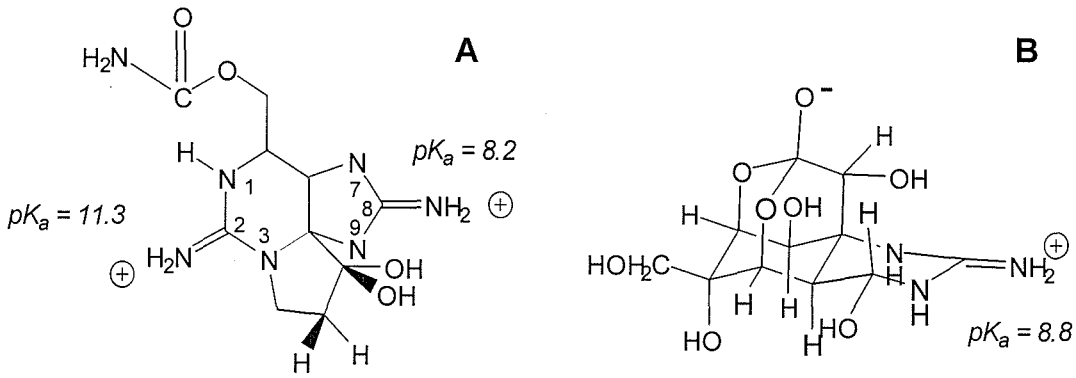


Figure 1.1 Structure of saxitoxin (A) and tetrodotoxin (B) illustrating the position of positively charged guanidinium moieties on each molecule.

1.2 Paralytic Shellfish Toxins

PSTs are produced by a variety of marine dinoflagellates and cyanobacteria and each species and strain produces a characteristic and complex mixture of toxins. Dense harmful algal blooms (HABs) of cyanobacteria can contaminate freshwater sources including dams, rivers, lakes, affecting both livestock and human health (Jones and Negri, 1997; Kaas and Henriksen, 2000; Negri et al., 1995).

In affected inland waters, PSTs accumulate in benthic feeders such as mussels which can impact on freshwater aquaculture and recreational fishing of affected species (Negri and Jones, 1995; Vasconcelos, 1999). Likewise, in coastal waters toxic dinoflagellate blooms can accumulate in filter feeders such as oysters and mussels (Blanco et al., 2003; Garate-Lizarraga et al., 2004; Oshima et al., 1987) and a variety of gastropod molluscs, crustaceans and fish (Chen and Chou, 2001; Ito et al., 2004; Llewellyn and Endean, 1989). Consumers of these vector species can also be at considerable risk with PSTs transmitted to higher vertebrates including humans, via the food web (Llewellyn et al., 2002; Rodrigue et al., 1990) and pose a major threat to the viability of both wild shellfish fisheries and shellfish farming operations.

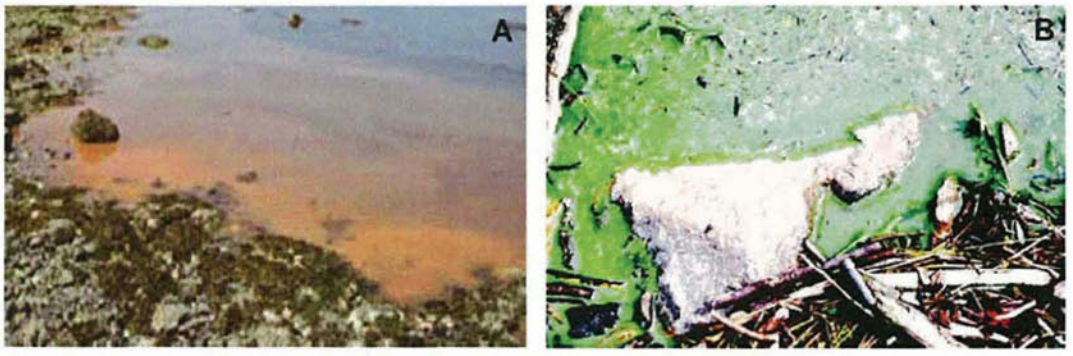


Figure 1.2 Photographs of toxic dinoflagellate (A) and cyanobacterial (B) blooms. Image (A) was obtained and modified from www.pac.dfo-mpo.gc.ca/ops/fm/shellfish/Biotoxins/PSP_e.htm and image (B) which illustrates a bloom of *Anabaena circinalis* (B) was from www.dlwc.nsw.gov.au.

PSTs are hydrophilic cyclic alkaloids and at least 24 known chemical STX derivatives have been reported (Hall et al., 1990; Negri et al., 2003b; Shimizu, 1993). STX selectively blocks the inward sodium current in excitable cells (Catterall, 1980; Denac et al., 2000b; Fozzard and Lipkind, 1996) causing a syndrome known as paralytic shellfish poisoning (PSP). One of the most toxic PST derivatives is the parent molecule, STX, which was reported to have a 50% lethal dose for mice (IP) in the order of 10 μg/kg (Carmichael, 1992). Based on this high potency, water solubility and reports of successful synthesis (Jacobi et al., 1984; Tanino et al., 1977), STX is recognised as a potential chemical weapon and is listed as a schedule one chemical alongside mustard and sarin gas by the

United Nations Chemical Weapons Convention (OPCW, 2002). PSP is characterised by neurological symptoms including dizziness, tingling and numbness in the face and extremities and gastrointestinal upset including nausea and vomiting due to a relaxant action on vascular smooth muscle cells (Falconer, 1993; Stommel and Watters, 2004; Watters, 1995). Onset of symptoms following consumption of contaminated shellfish or water have been reported to take from between 30 minutes and 12 hours (Lehane, 2000; Smart, 1995; van Dolah, 2000) and in severe intoxications, muscular paralysis, respiratory paralysis and death may result (Cheng et al., 1991; Llewellyn et al., 2002; Rodrigue et al., 1990). At present there is no antidote or treatment for PSP but supportive care such as artificial respiration is crucial for patient recovery (Stommel and Watters, 2004), allowing time until the toxins are eliminated from the body by renal clearance (Andrinolo et al., 2002; Andrinolo et al., 1999; Gessner et al., 1997).

1.3 Sources of Paralytic Shellfish Toxins

In marine systems, PSTs naturally occur in a variety of bloom forming dinoflagellates from the genus *Alexandrium*, *Pyrodinium* and *Gymnodinium* (Table 1.1) and in freshwater environments, certain filamentous cyanobacteria including *Anaebaena, Amphizomenon, Cylindrospermospsis, Lyngbya* and *Planktothrix* species have been reported to produce the deadly toxins (Table 1.1). Of these known PST producers, only six have been reported in Australian waterways including five marine dinoflagellates and one representative of the blue green alga, *Anabaena circinalis* (Humpage et al., 1994); see Fig. 1.2B). The global occurrences of saxitoxic cyanobacterial (Kaas and Henriksen, 2000; Pereira et al., 2000; Pomati et al., 2000) and dinoflagellate blooms (Hallegraeff, 1993; Quilliam, 2001) has been increasingly reported (Hallegraeff, 1993; Quilliam, 2001). The biological advantage of PST production in these sources has not been clarified but considerable efforts have been made towards the understanding of the biosynthetic pathways, metabolism and molecular mechanisms of these secondary metabolites in cyanobacteria and dinoflagellates (Pomati et al., 2001; Pomati et al., 2004b; Pomati and Neilan, 2004; Shimizu, 1986a; Shimizu, 1986b; Shimizu, 1988; Shimizu, 1996).

Table 1.1 Reported dinoflagellates and cyanobacterial sources of paralytic shellfish toxins in the marine and freshwater environment.

Marine		Freshwater	
Species	Reference	Species	Reference
Dinoflagellates		**Cyanobacteria**	
Alexandrium andersoni	(Climiniello et al., 2000)	*Anabaena circinalis**	(Humpage et al., 1994)
*A. catenella**	(Negri et al., 2003a)	*Anabaena lemmermannii*	(Kaas and Henriksen, 2000)
A. excavatum	(Cembella et al., 1993)	*Aphanizomenon flos-aquae*	(Mahmood and Carmichael, 1986)
A. fundyense	(Anderson et al., 1990)	*Aphanizomenon issatschenkoi*	(Nogueira et al., 2004)
*A. minutum**	(Hallegraeff et al., 1988)	*Aphanizomenon gracile*	(Pereira et al., 2004)
A. ostenfeldii	(MacKenzie et al., 1996)	*Lyngbya wollei*	(Carmichael et al., 1997)
*A. tamarense**	(Oshima et al., 1992)	*Cylindrospermopsis raciborskii*	(Lagos et al., 1999)
A. tamiyavanichii	(Kodama et al., 1988a)	*Planktothrix sp.*	(Pomati et al., 2000)
*Gymnodinium catenatum**	(Oshima et al., 1987)		
Pyrodinium bahamanse var compressa	(Harada et al., 1982)		

* Known occurrence in Australia according to (Negri et al., 2003a)

Several studies have suggested that PSTs may also be produced by bacteria associated with dinoflagellate cultures, including *Moraxella sp.* (Kodama et al., 1990; Kodama et al., 1988b) and *Alteromonas sp.* and *Pseudomonas spp.* (Gallacher et al., 1997; Gallacher et al., 1996; Gallacher and Smith, 1999). In addition, several marine bacteria from shellfish including *Vibrio spp.* and *Pseudomonas spp.* (Gallacher and Smith, 1999; Levasseur et al., 1996) and even enterobacter bacteria from the rumen of cattle (Sevcik et al., 2003) have been reported to produce STXs. However, levels of PST production by both symbiotic and free living bacteria in each of these cases, does not account for the total toxin concentrations observed in dinoflagellates and chemical identification of STX derivatives has not been unambiguously confirmed. In fact, one report clearly demonstrated that several *Pseudomonas spp.* bacteria isolated from *Alexandrium spp.* produced fluorescent components that mimicked STX peaks during High Performance Liquid Chromatography (HPLC) analysis (Baker et al., 2003b). Nevertheless, mounting evidence on the effects of isolated bacteria in the bio-tranformations, elimination and metabolism of PSTs from both dinoflagellates and benthic animals (Geier, 2003; Kotaki et al., 1985; Smith et al., 2001), suggests that bacteria may have a contributory role in toxin pharmacokinetics. A discussion

of sources and vectors identified and distributed in Australian waters is provided in Chapter 4.

1.4 Chemistry and Toxicity

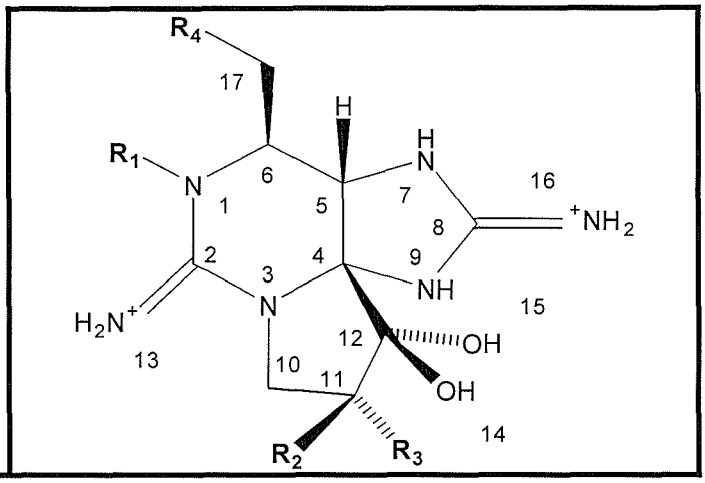
STX was the first isolated PST derivative and was purified from the Alaskan butter clam, *Saxidomus giganteus* from which its name was derived (Schantz et al., 1957). It was not until more than 15 years later that the crystal structure of STX, also isolated from *S. giganteus* was elucidated (Schantz et al., 1975). The isolation of STX on carboxylate resins (e.g. BioGel P2 and BioRex 70) which was devised in these early years (Oshima et al., 1977; Shimizu, 1985) are still used routinely and efficiently for the purification of PSTs (Lippemeier et al., 2003; Negri et al., 1997). Each PST is characterised by a unique tricyclic ring system with two guanidinium groups at either side of the molecule (see Fig. 1.1). The pyrimidine guanidine has a highly basic pKa of approx. 11.3 for STX hydrate while the imidazole guanidine has a much lower pKa of approx. 8.2 (Rogers and Rapoport, 1980). Another key feature of the PSTs is the presence of a hydrated ketone at C12 (see structure in Table 1.2), which is also a critical element in mammalian toxicity (Hu et al., 1987; Onodera et al., 1997). At neutral pH these hydroxyl groups are protonated and have been correlated to an increase in the toxic action of these compounds (Hall and Reichardt, 1984).

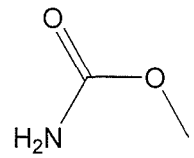
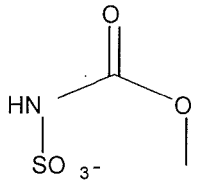
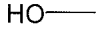
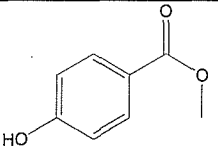
The PSTs form a group of closely related derivatives which can be divided into five groups according to their structural differences (see Table 1.2). These groups include the i) carbamate (STX, neoSTX and gonyautoxins (GTX1-4)); ii) N-sulpho-carbamoyl (GTX5-6, C1-4); iii) decarbamoyl (dc-) (dcSTX, dcneoSTX, dcGTX1-4); iv) deoxydecarbamoyl (do-) (doSTX, doneoSTX and doGTX1); and (V) hydroxybenzoates (GC1-3).

Each PST group differs in relative mammalian toxicity with the non-sulphated STX and neosaxitoxin (neoSTX) being the most potent, followed by the singly sulphated gonyautoxins (GTXs), and the mildly toxic C toxins (Oshima, 1995a) see Table 1.2). In natural sources of PSTs such as the dinoflagellate, *A. minutum*, a complex mixture of these derivatives may be present. These toxin profiles have been reported to have characteristic signatures depending on species, strains and

even geographical locations (Chang et al., 1997; MacKenzie et al., 1996; Taleb et al., 2003) which can be useful in determining the likely causative organism.

Table 1.2 Structure and toxicity of known paralytic shellfish toxins.



PST	Toxicity† MU/µmol	R1	R2	R3	R4
STX	2483	H	H	H	
GTX2	892	H	H	OSO_3^-	
GTX3	1584	H	OSO_3^-	H	
NEO	2295	OH	H	H	
GTX1	2468	OH	H	OSO_3^-	
GTX4	1803	OH	OSO_3^-	H	
GTX5	160	H	H	H	
C1	15	H	H	OSO_3^-	
C2	329	H	OSO_3^-	H	
GTX6	-	OH	H	H	
C3	33	OH	H	OSO_3^-	
C4	143	OH	OSO_3^-	H	
dcSTX	1872	H	H	H	
dcGTX2	1617	H	H	OSO_3^-	
dcGTX3	1872	H	OSO_3^-	H	
dcNEO	-	OH	H	H	
dcGTX1	-	OH	H	OSO_3^-	
dcGTX4	-	OH	OSO_3^-	H	
doSTX	-	H	H	H	
doGTX2	-	H	H	OSO_3^-	
doGTX3	-	H	OSO_3^-	H	
GC3	-	H	H	H	
GC1	-	H	H	OSO_3^-	
GC2	-	H	OSO_3^-	H	

† Toxicity values reported by (Oshima, 1995a)
- Not tested

However, in some locations this differentiation of strains and even species has proved challenging (Chou et al., 2004), which is not surprising when toxicity and PST profiles of certain species may be affected by changes in growth conditions (Hwang and Lu, 2000; Hwang et al., 2003; Lippemeier et al., 2003). The discovery of genetic markers and molecular methods for identification of toxic species and strains is currently in rapid development (Galluzzi et al., 2004; Godhe et al., 2001; Guillou et al., 2002; Pomati et al., 2004a; Pomati and Neilan, 2004) and the potential for both sensitive detection and accurate identification of toxic algae by these methods is likely.

The majority of PSTs exist in ionised forms, are heat stable at acidic pH, are highly soluble in water, but insoluble in lipid solvents (Hall et al., 1990). These qualities are significant in terms of seafood safety because the act of cooking contaminated shellfish would not be likely to eliminate the toxicity. In fact, cooking may increase toxicity by hydrolysing any mildly toxic *N*-sulphocarbamoyl derivatives (e.g. C 1-4, GTX 5, GTX 6), commonly found in a variety of Australian cyanobacteria and dinoflagellate species, to a desulphated form (GTX, STX, neoSTX). This has been demonstrated to cause dramatic increases in mouse toxicity (Hall and Reichardt, 1984). In alkaline conditions, PSTs are highly unstable and are easily oxidised and degraded which suggests that they would degrade in seawater (approx. pH 8) unless complexed with other stabilising molecules (Shimizu, 2000).

1.5 Voltage Gated Sodium Channels

Consumption of PST contaminated shellfish can have marked physiological effects due to the rapidly reversible blockade (sec.- min.) of the VGSC at the neuromuscular junction in excitable cells (Denac et al., 2000b). This blockade occurs at the extracellular side of the plasma membrane and impedes Na^+ influx into the cell which is critical for depolarisation of the membrane and subsequent propagation of action potentials. The rapid onset of parasthesias, numbness and muscular and respiratory paralysis associated with PSP syndrome can be explained by this high affinity binding (dissociation constant K_d ~1-5 nM), with a single toxin molecule interacting with a single VGSC (Campbell and Hille, 1976; Moczydlowski et al., 1984).

STX, TTX and their analogues have proved to be valuable tools in the physiological understanding of VGSCs and have greatly facilitated examination of the shape and structure of the outer vestibule and in modelling of the TTX and STX binding site (Lipkind and Fozzard, 1994). Unfortunately due to the large size (approx. 300 kDa) and complexity of this trans-membrane protein (Sato et al., 1998), the complete crystal structure of the VGSC is yet to be successfully elucidated, but extensive modelling from the encoded cDNA sequences and pharmacological examination of individually cloned subunits has provided a wealth of information on the structure and interaction of each subunit. In rat brain, the VGSC is reported to be a heterotrimeric protein containing subunits α, β1 and β2, (see Fig. 1.3) whereas in heart and skeletal muscle only the α and β1 subunits are present (Roberts and Barchi, 1987; Satin et al., 1992a). The α-subunits of many different species have now been cloned and sequenced revealing highly conserved regions of nucleotide sequence (Chen et al., 1997; Noda et al., 1986; Rogart et al., 1989; Satin et al., 1992b). The α-subunit has four repeating units, each consisting of six alpha-helical trans-membrane segments (see Fig. 1.3) which are thought to be arranged in a clockwise configuration around the central ion permeation site (Dudley et al., 2000; Li et al., 2000). The positively charged guanidinium groups of PSP toxins (see Fig. 1.1) interact with negatively charged carboxyl groups (Khan et al., 2002) on the P-loops situated at the mouth of the channel between segments 5 and 6 of the α-subunit (see Fig. 1.3). Point mutation studies revealed that several glutamate residues corresponding to these P-loops are essential for effective inhibition of Na^+ current by STX and included Glu 387 (Noda et al., 1989), Glu 942, Glu 945 and Asp 384 (Lipkind and Fozzard, 1994; Satin et al., 1992a; Terlau et al., 1991). Binding studies with tritiated STX also suggest that the β1-subunit participates in forming the TTX/STX binding site (Messner and Catterall, 1986).

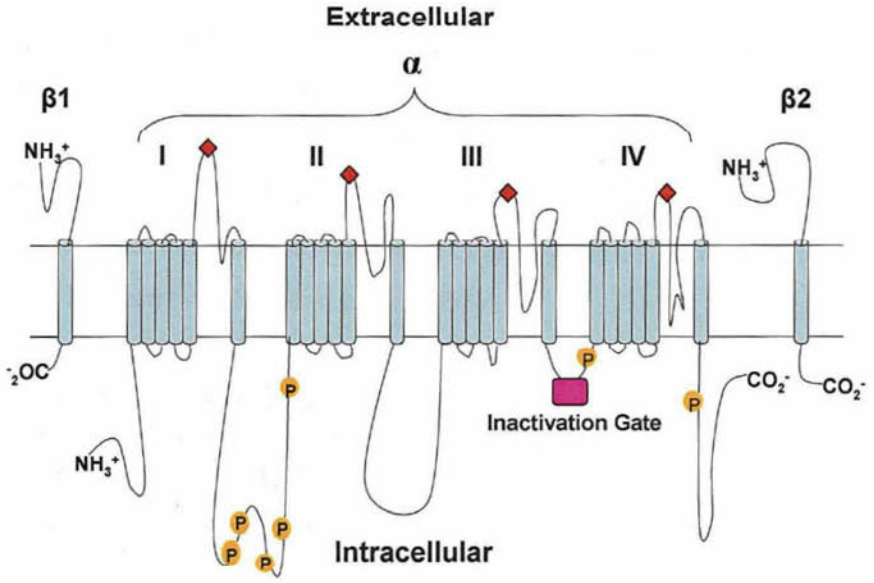


Figure 1.3 Schematic model of the secondary structure of the voltage gated sodium channel highlighting the arrangement of alpha helices within the phospholipid membrane. The alpha subunit consists of 4 homologous domains (I-IV) with 6 transmembrane segments and two beta subunits (β1, β2). The polypeptide chain is represented by the solid line and cylinders represent α-helices. Red diamonds on the extracellular surface of the membrane represent the sites that are thought to interact with the positively charged guanidino groups on the STX molecule. Orange circles indicate sites of demonstrated protein phosphorylation and the inactivation gate is highlighted in pink. Schematic is based on model by Lipkind and Fozzard 1994.

1.6 Regulatory Limits and Detection of PSTs

PSTs pose a serious public health threat due to their high potency and potential contamination to food and water supplies. In most countries, commercial shellfish harvesting is now regulated and levels of PSTs monitored closely to prevent contaminated stock reaching consumers (van Egmond et al., 1992). At present most monitoring programs only require that filter feeding shellfish such as clams, mussels and oysters are tested routinely for PST contamination. However, reports of PSTs in animals higher in the food chain such as carnivorous gastropods (Hwang et al., 1994; Ito et al., 2004) and crustaceans (Baden, 1983; Bretz et al., 2002), emphasises the importance of additional classes being considered for testing prior to reaching consumers (Shumway, 1995).

The most widely accepted safe level of PST contamination is presently 80 μg STX equivalents/ 100 g tissue (see Table 1.3), which is not particularly conservative considering that illness has been reported at PSP doses as low as 144

μg per person and fatalities have been reported at 300 μg (van Egmond et al., 1993). A single meal could easily reach these levels given that an average meal may consist of a 200-300 g serving of shellfish potentially contaminated with STX at the regulatory limit. If the lowest reported toxic dose of STX was used to establish a tolerance level, the current regulatory limits would need to be substantially reduced (van Egmond et al., 1993).

Table 1.3 Regulatory limits for the safe consumption of shellfish required by various countries around the globe. Table was adapted from data reported by Lehane (2000).

Country	Regulatory PST level (μg STXeq/ 100g tissue)[†]
Australia	80
New Zealand	80
USA	80
Norway	36
Japan	72
European Union	80
Canada	80

[†] STXeq= saxitoxin equivalents; 0.18 μg STXeq/ 100g tissue = 1MU

The most widely accepted method of detection of PSTs from shellfish is the mouse lethality assay which was established from the early work of Sommer and coworkers (Sommer and Meyer, 1937; Sommer, 1937). This was the first bioassay that made it possible to quantify the toxicity of STX and all of its analogues. The mouse bioassay is a standardised procedure in which the strain, size and condition of the mice are all critical to the accuracy of the toxicity calculation (AOAC-International, 1997). The basic protocol suggested by the Association of Official Analytical Chemists (AOAC) involves injecting an acid extract of shellfish (1mL) into the mouse (20 g) and then recording the time of death at the last breath. Standard dose response curves are constructed using calibrated STX standards and toxic sample extracts are diluted to ensure mortality within five to 15 minutes (AOAC-International, 1997). The AOAC method is still the dominant method used for detecting PSTs despite having a detection limit of only 40 μg STXeq/100 g shellfish, which is quite poor compared to other methods that are available (see Table 1.4) and for these reasons is often coupled to analytical methods such as HPLC (Asp et al., 2004; van Egmond et al., 2004). The major drawbacks of the

mouse lethality assay centre around the use of mice, which not only have considerable ethical considerations but need to be maintained in constant stocks weighing 19-22 g which can be labour intensive and expensive (AOAC-International, 1997; Lehane, 2000; van Egmond, 2004). The effects of the PSTs on the mice are both strain and colony dependent, which means that large numbers of mice are needed from each new colony, to produce a single dose response curve from which sample extracts can then be tested. Other reported problems inherent with this assay includes interference of salt which can suppress toxic effects at high concentrations (LeDoux and Hall, 2000) and extracts of oysters contaminated with zinc, have been reported to cause lethal effects even when sub-lethal doses of PSTs have been injected (Aune et al., 1998).

Many alternative PST detection methods have been developed including the *in vivo* locust lethality assay (McElhiney et al., 1998); *in vitro* cell based (Jellett et al., 1992; Kogure et al., 1988), hippocampal slice (Kerr et al., 1999) and sodium channel assays (Doucette et al., 1997; Ruberu et al., 2003); biochemical methods such as enzyme-linked immunosorbent assays (ELISA) (Chu and Fan, 1985; Chu et al., 1996; Usleber et al., 2001) and centipede SXPN assay (Llewellyn et al., 2001a; Llewellyn et al., 1998); chemical analysis by HPLC (Oshima, 1995a; Oshima, 1995b; Sullivan and Iwaoka, 1983), capillary electrophoresis (Pleasance et al., 1992; Quilliam, 1995) and mass spectrometry (Dahlmann et al., 2003; Pleasance et al., 1992; Quilliam et al., 1989). Each method has inherent advantages and disadvantages with varying degrees of sensitivity to PSTs, but all eliminate the use of mammals and have lower detection limits that the mouse bioassay (see Table 1.4). In general, there is a distinct need for a detection method that meets several criteria:

- Sensitive to all PST derivatives
- Accurate and low detection limits
- Robust to the effects of salt, metals and other contaminants
- Simple and inexpensive
- Correlates well to mammalian toxicity
- Does not require specialist equipment or training

Table 1.4 Comparison of a variety of reported methods for the detection of PSTs.

Detection Method	STX Detection Limit† (μg /100g)	Advantages	Disadvantages	References
Mouse Mortality Bioassay	35-40	Closely predicts human toxicity, sensitive to all derivatives	Ethical considerations; costly; time consuming, high variability; cannot confirm toxin identity or profile.	(Adams and Miescier, 1980; Salter et al., 1989)
MIST-Alert Cell Bioassay Kit	2	Simplified cell bioassay, elimination of animals and radioligand, sensitive to all toxins	Expensive; limited shelf-life; cannot confirm toxin identity or profile.	(Jellett et al., 2002; Llewellyn et al., 2001a; Mackintosh et al., 2002)
RidaScreen STX ELISA	0.25	Rapid, simple, sensitive	Dependent on antibody specificity; cannot confirm toxin identity or profile.	(Usleber et al., 2001)
Sodium Channel Assay	0.2- 1	High-throughput, Sensitive to all derivatives	Requirement of rat brain; reliance on radioligand; cannot confirm toxin identity or profile.	(Doucette et al., 1997; Llewellyn et al., 2001b; Ruberu et al., 2003)
Centipede Saxiphilin Assay	1- 2	High-throughput, Sensitive, distinguishes between TTX and STX-like activity	Centipede availability is limited; reliance of radioligand; cannot confirm toxin identity or profile; Low sensitivity to C-toxins.	(Llewellyn and Doyle, 2001; Negri and Llewellyn, 1998)
Liquid chromatography	0.01-1	High sensitivity, confirmation of toxin identity and profile.	Specialised equipment and training; requires calibrated toxin standards.	(Lawrence et al., 1991; Negri and Llewellyn, 1998; Oshima, 1995a)
Mass Spectrometry	0.001-1	High sensitivity, structural confirmation of toxin identity and profile.	Specialised equipment and training	(Pleasance et al., 1992; Quilliam, 2003; Quilliam et al., 1993)

Note: This list is not exhaustive and so does not include all reported methods available.
† For comparison reported detection limits were converted to μg STX eq.

1.7 Saxitoxin Resistance

Fossil evidence of dinoflagellate cysts suggest that these organisms have existed for at least a billion years (Graham and Wilcox, 2000) and are thought to be derived from a endosymbiotic event between a cyanobacterium and eukaryotic organism (Mordon and Sherwood, 2002). Based on its unique distribution as a bioactive secondary metabolite in both prokaryotes and eukaryotes, it has been

suggested that STX may have been acquired early in the evolution of these micro-organisms (Shimizu, 1993). Reports of the red tide phenomenon and their toxic effects have been recognised for centuries and references in the Old Testament of the Bible are thought to relate to a dinoflagellate bloom in the Nile River, an event that has been dated back to approximately 1500BC by both archaeological and historical data (Rohl, 1999).

> *Exodus 7:20-21: ".....and the waters in the river were turned to blood. And the fish in the river died; and the river became foul smelling, and the Egyptians could not drink its water."*

In light of this history of occurrence, it is not surprising that many benthic animals have developed mechanisms of resistance to some of the toxins produced by cyanobacteria and dinoflagellates. Several suggested mechanisms of STX resistance have been reported including nerve insensitivity (Daigo et al., 1988; Kvitek and Beitler, 1991), STX induced proteins (Barber et al., 1988; Smith et al., 1989) and soluble STXBPs (Llewellyn, 1997). Each of these mechanisms, which have been observed in a variety of species, may afford the exposed animal protection from PST intoxication.

Marine species that have demonstrated resistance to STX generally accumulate this toxin at high concentrations without detriment as was demonstrated in the Xanthid crab *Atergatis floridus* which is a well recognised vector of PSTs (Arakawa et al., 1995; Raj et al., 1983; Tsai et al., 1995) and has a minimal lethal dose of 5,000-10,000 MU/ 20 g, equivalent to approximately 4500-9000 μg STX/ 100 g (Koyama et al., 1983). Crab resistance has been correlated with detectable levels of PSTs in a variety of crab species (Koyama et al., 1983; Yasumoto et al., 1981) and resistance has been attributed to the presence of PST insensitive nerves (Daigo et al., 1988). Similar effects were observed in the mussel *Mytilus edulis* and a variety of other bivalve molluscs which accumulated large quantities of PSTs but had no observed reaction to the toxins unlike less toxic species (Twarog, 1974). Further studies from these animals demonstrated that resistance to STX was also due to PST insensitive nerves (Kvitek and Beitler, 1991; Twarog, 1974). In contrast, marine species susceptible to the effects of STX do not accumulate

excessive concentrations within their tissues and usually demonstrate typical PSP symptoms when exposed to PSTs (Adams et al., 1968; Koyama et al., 1983; Smith et al., 1989; Twarog, 1974).

Not all marine animals exposed to STX have nerves that are resistant to PSTs yet are able to survive and accumulate moderate levels of PSTs in these environments. For instance, the appearance of a STX induced protein in the crab, *Hemigrapsus oregonesis* following STX injection was correlated to resistance in this species (Barber et al., 1988). Further investigation of the anti-STX induced protein in extracts of a selection of bivalves suggested that a related protein may be present in those species (Smith et al., 1989). These proteins which were reported more than fifteen years ago has never been sequenced or fully characterised so the true nature of its functional relationship to PSTs or STX binding capability is not known. In another study hemolymph from several Xanthid crabs including *Lophozozymus pictor, Liomera tristis, Chlorodiella nigra* and *Actaeodes tomentosus*, were reported to contain STX-specific binding activity and it was suggested that the component involved may act as a STX resistance mechanism in these animals (Llewellyn, 1997; Llewellyn et al., 1997). However, as with nerve resistance, this STX-binding hemolymph factor was not present in known toxic species and in all non-toxic species and presence of the binding factor did not seem to correlate with increased PST accumulation (Llewellyn, 1997), although this was not conclusively examined.

STX resistance in terrestrial species has also been reported. One early report concluded that frogs were resistant to the effects of natural toxins contained in crude mussel extracts compared to mice and demonstrated a lethal dose 15 times that required to cause mouse mortality being required to induce PSP symptoms in frogs (Prinzmetal et al (1932) as reported by Mahar et al (1991)). This resistance was confirmed in a subsequent study of the effects of pure STX in the leopard frog *Rana pipiens* (Kao and Fuhrman, 1967). A minimal lethal dose of 75 μg/kg was required in the frog compared to only 5 μg/kg in mice tested at that time, while the effect of TTX in both animals was similar (Kao and Fuhrman, 1967). Unlike several crab and mollusc species, this resistance in frogs could not be correlated to STX-insensitive nerves which have been clearly shown to bind both

STX and TTX with high affinity (Campbell and Hille, 1976; Hille et al., 1975; Strichartz, 1984b). It has been postulated that the presence of SXPN, a 91 kDa soluble STXBP isolated from *Rana catesbeiana,* is responsible for the reduced effects of STX that have been observed in a variety of amphibian species (Mahar et al., 1991). Similar STXBP activity has since been demonstrated in a variety of ectothermic vertebrates including fish, reptiles and amphibians and in a small number of invertebrates including several crustacean, insect and arachnid species (Llewellyn, 1997; Llewellyn et al., 1997; Mahar et al., 1991). The phylogenetic distribution of soluble STX binding activity is discussed in detail in Chapter 3.

1.8 Soluble Saxitoxin Binding Proteins

Soluble STX binding activity from biological sources was first recognised in the clarified supernatant of frog heart homogenates (Doyle et al., 1982; Tanaka et al., 1984), an activity which was confirmed in an independent investigation of the distribution of VGSC subtypes in frog skeletal muscle (Moczydlowski et al., 1988). From these early observations, a distinct class of binding sites were isolated from soluble muscle extracts of the North American Bullfrog, *Rana catesbeiana,* and had a high affinity (0.1 nM) for [^{3}H] STX but was insensitive to TTX (Moczydlowski et al., 1988) and on this basis was deemed pharmacologically distinct from the VGSC. STX binding activity was attributed to a soluble plasma protein, which was aptly named SXPN based on its binding specificity for STX (Mahar et al., 1991). A wealth of data has been reported for *R. catesbeiana* SXPN and the amino acid sequence from molecular cloning studies revealed a high homology for the transferrin (TF) family of iron binding proteins (Li and Moczydlowski, 1991; Morabito and Moczydlowski, 1994; Morabito and Moczydlowski, 1995) which is discussed further in section 1.9.

Another class of soluble STXBPs was recently reported and was isolated from the plasma of pufferfish, *Fugu pardalis* (Yotsu-Yamashita et al., 2001). The soluble nature of these proteins distinguished them from the VGSC, however the demonstrated co-affinity for TTX (K_d~ 12 μM) and STX (K_d~ 8.5 nM) suggested that the proteins were also dissimilar to SXPN (Yotsu-Yamashita et al., 2002). Subsequent cloning and amino acid sequencing revealed that these proteins, named PSTBPs, were most similar to the partially sequenced TTX binding

proteins (TTXBP), isolated from related pufferfish, *Takifugu niphobles* (Matsui et al., 2000).

1.9 Saxiphilin

SXPN is a 91 kDa plasma protein isolated from *R. catesbeiana* (Morabito and Moczydlowski, 1994). [^{3}H] STX specifically binds to a single site on the SXPN molecule with high affinity (K_d= 0.18 nM) and has varied affinity for other PSTs (Llewellyn and Moczydlowski, 1994; Mahar et al., 1991) and is discussed further in Chapter 3. SXPN does not interact or bind TTX (Moczydlowski et al., 1988), unlike the membrane-bound voltage gated sodium channels (VGSC) (Denac et al., 2000a) and puffer-fish STX and TTX binding proteins (PSTBPs) (Yotsu-Yamashita et al., 2002; Yotsu-Yamashita et al., 2001). Pharmacological characterisation studies revealed that SXPN binding of [^{3}H] STX to SXPN is partially inhibited by a variety of divalent metal and lanthanide cations and the carboxyl-methylating reagent trimethyloxonium (Llewellyn and Moczydlowski, 1994). The latter activity indicated that at least one carboxyl moiety is likely to be required within the STX binding on the molecule and it was postulated that this site was located in the C-terminus of the protein (Llewellyn and Moczydlowski, 1994).

The unique STX binding activity of the protein facilitated initial purification efforts which incorporated assay guided fractionation of crude plasma by size exclusion chromatography (SEC), cation exchange chromatography (CEX; S-Sepharose) and isoelectric focussing (Mahar et al., 1991). While these trials resulted in 70 % loss of activity, estimates of size (74 ± 8 kDa), basic isoelectric point (approx. 10.5) and net positive charge of the molecule at pH 6, were crucial for the development of a successful SXPN purification strategy (Mahar et al., 1991). Final purification was achieved by Heparin Sepharose affinity chromatography and chromatofocussing with PBE 94 anion exchanger gel (Li and Moczydlowski, 1991). This methodology resulted in a 436 fold purification of SXPN from plasma and produced a single Coomassie stained band following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with an estimated molecular weight of approx. 89 kDa (Li and Moczydlowski, 1991).

Purification and partial sequencing of native SXPN from bullfrog plasma identified a possible relationship between SXPN and TFs (Li and Moczydlowski, 1991) and facilitated the isolation of cDNA encoding SXPN (Morabito and Moczydlowski, 1994; Morabito and Moczydlowski, 1995). Further immunochemical and biochemical evidence demonstrated that TF was in fact distinct from SXPN based on differences in size (~78 kDa TF; 89 kDa SXPN), chromatographic behaviour (DEAE Sepharose), visible absorption spectra and ligand binding (Li et al., 1993). The translated protein sequence of SXPN showed high amino acid sequence homology (30-70 % identity) to the TF family of Fe^{3+} binding proteins (Morabito and Moczydlowski, 1994; Morabito and Moczydlowski, 1995).

Transferrins are a family of single-chain, glycosylated proteins which include serum transferrin (ST), lactoferrin (LT), melanotransferrin and (MTF), inhibitor of carbonic anhydrase (ICA) and ovotransferrin (OT) (for review see (Aisen and Leibman, 1972; Baker et al., 2002; Lambert et al., 2005). TFs are iron transport molecules which rapidly and efficiently mobilise iron from plasma to cells and regulate plasma iron levels. The majority of TFs consist of two homologous lobes (N and C) connected by a short hinge region (see Fig 1.4A; (Baker et al., 2002). Each of the two lobes is able to reversibly bind a single Fe^{3+} ion and an associated carbonate ion (HCO_3^-; see Fig 1.4B).

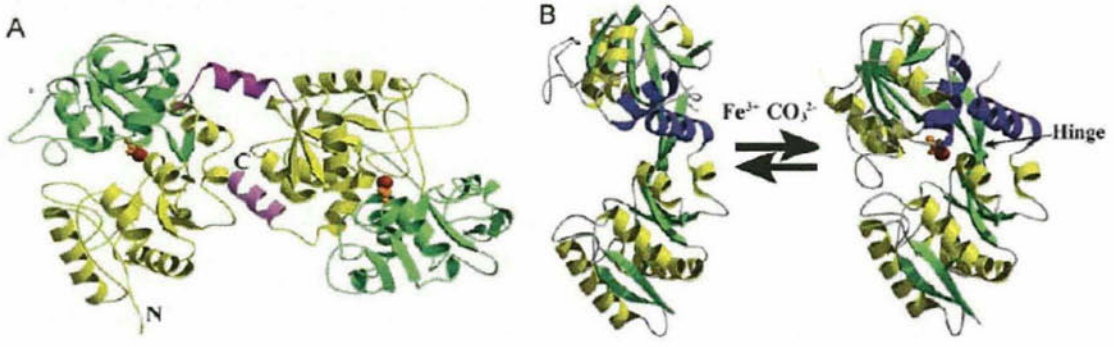


Figure 1.4 Characteristic ribbon structure of transferrins obtained and from Baker, 2003. (A) The iron-bound form of human lactoferrin illustrating the bilobed nature of the molecule which is common to transferrins and position of Fe^{3+} (red) and associated CO_3^{2-} (orange) molecules. (B) The conformational change which occurs during iron-binding shown in the N-lobe of human transferrin.

The binding interaction between Fe^{3+} and HCO_3^- has been described as synergistic and binding studies have demonstrated that Fe^{3+} cannot effectively bind to TFs without the concomitant binding of HCO_3^- and vice versa (Baker et al., 1996). In fact both kinetic and structural data suggests that HCO_3^- is required to bind to TF first to facilitate Fe^{3+} binding (Anderson et al., 1989; Baker et al., 1996). Fe^{3+}-bound TF is internalised by binding to a cell surface receptor and is then propelled into the endosome compartment of the cell by endocytosis. The acidic environment of the endosome induces the release of Fe^{3+} allowing recycling of apotransferrin (TF minus Fe^{3+}) to the cell surface (Baker et al., 2003a; Baker and Baker, 2004).

Full amino acid sequencing of SXPN revealed that SXPN differs from TFs with substitutions at 9 of 10 highly conserved residues which are known to be directly involved in the two Fe^{3+}/HCO_3^- binding sites (Morabito and Moczydlowski, 1994). These differences can be readily demonstrated by multiple protein sequence alignment with other TFs (see Fig 1.5). These differences are quite significant in terms of function and may account for the inability of SXPN to bind Fe^{3+} (Li et al., 1993).

Another interesting homology, unrelated to TF, was the unique 143 amino acid insertion in the N-lobe of SXPN (see Fig 1.6). This insertion contains two homologous thyroglobulin type-1 domains (Thyr-1), which is unanimously absent in TFs but are present in diverse protein families including mosaic proteins such as thyroglobulin, membrane proteins such as the p41 invariant chain and insulin-like growth factor- binding proteins (Lenarcic and Turk, 1999; Shimasaki et al., 1991; Turk et al., 1999; Yamashita and Konagaya, 1996). Thyr-1 modules have been shown to inhibit the papain family of cysteine proteinases (Galesa et al., 2003; Lenarcic and Turk, 1999; Pungercic et al., 2002; Turk et al., 1999; Yamashita and Konagaya, 1996) and their presence in SXPN explains the potent inhibition of papain, human cathepsin B and cathepsin L that has been recently demonstrated (Lenarcic et al., 2000).

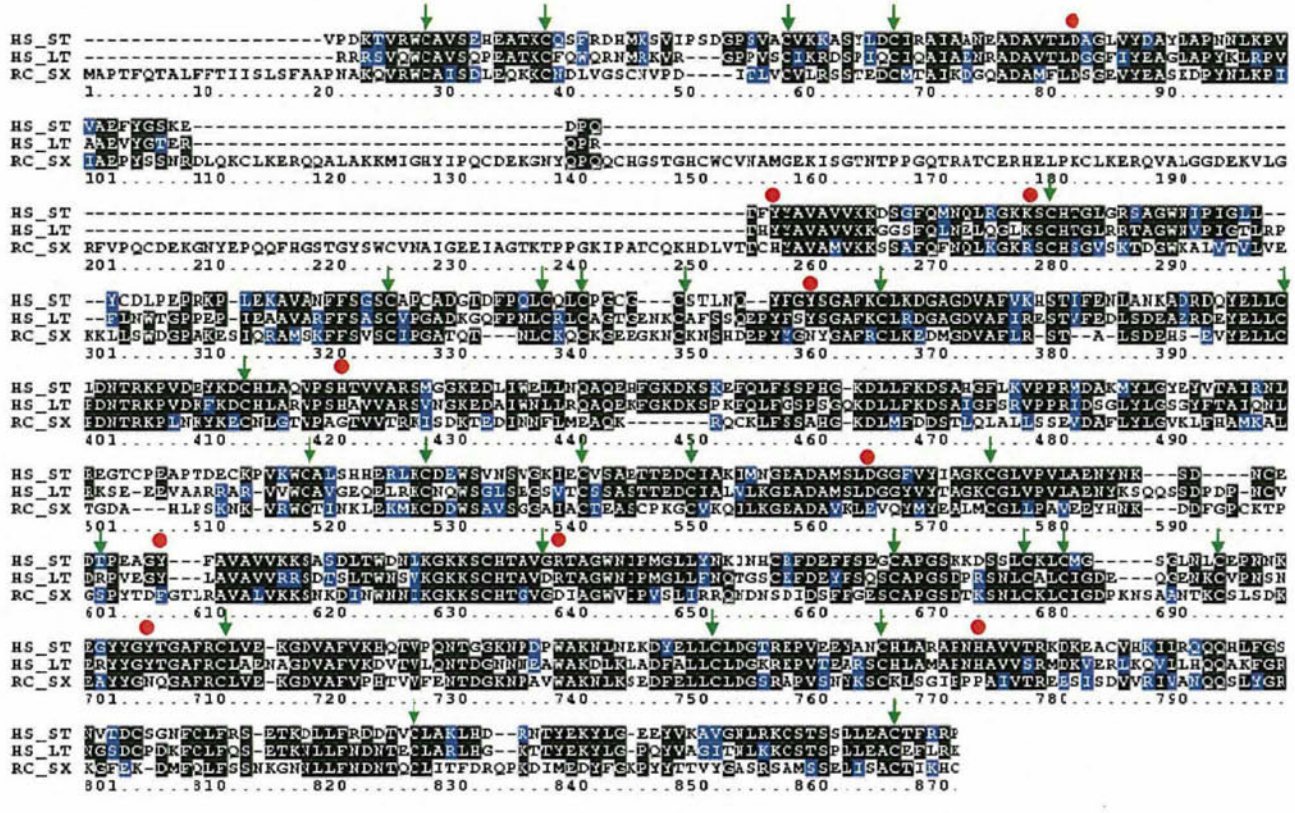


Figure 1.5 Protein sequence alignment of *Rana catesbeiana* saxiphilin (RC_SX), human lactoferrin (HS_LF) and serum transferrin (HS_ST). The alignment was produced using CLUSTALW with BioManager hosted by Australian National Genomic Information Service. Gaps in the alignment appear as hyphens (-). Identical residues are highlighted in black and similar residues are in blue. The positions of 10 highly conserved residues of the Fe^{3+}/ HCO_3^- sites from the N and C lobe of transferrins are indicated red circles and green arrows indicate conserved cysteine residues suggested to form disulphide bonds. Sequence highlighted in pink corresponds to a region of 143 amino acids from saxiphilin which is absent in transferrins. Figure based on sequence data presented in Morabito *et al* 1994, 1995.

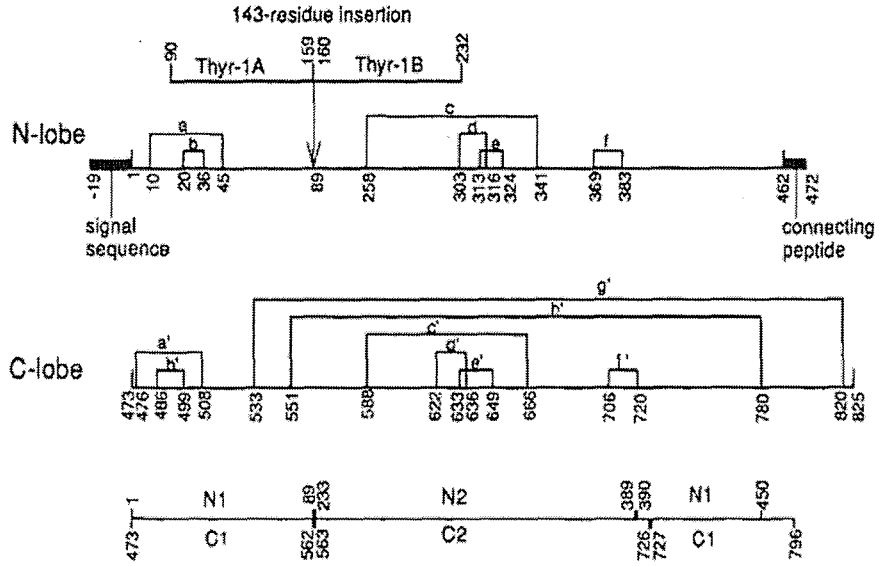


Figure 1.6 Schematic representation of the linear sequence of bullfrog saxiphilin (SXPN) showing the location of conserved disulphide bonds and N- and C-lobe structural domains predicted on the basis of sequence alignment with human lactoferrin as presented by Morabito *et al* (1995). Numbers refer to the SXPN amino acid sequence (Morabito & Moczydlowski, 1994, 1995) and residues pairs a-f and a'-h' indicate predicted disulphide bonds. SXPN residues 90-232 containing two type 1 thyroglobulin domains (Thyr-1A and 1B) are shown as an insertion in the N-lobe. The bottom line shows the relative location of SXPN sequences corresponding to predicted sub-domains of the N-lobe and C-lobe, respectively. Figure taken from Morabito *et al* 1995.

Further structural information on bullfrog SXPN was obtained following functionally expression and characterisation with a baculovirus expression (BVE) vector (Krishnan et al., 2001; Morabito et al., 1995). These studies demonstrated that the recombinant SXPN had retained functional activity and thus protein folding consistent with the native protein (Morabito et al., 1995). A BVE vector was also constructed, encoding a 361 amino acid residue insert, homologous to the C-lobe of TFs (Morabito et al., 1995). This recombinant C-lobe of SXPN demonstrated low affinity [^{3}H] STX binding activity (0.9 nM) with a faster dissociation rate and pH dependence closely resembling native SXPN (see Table 1.5) (Llewellyn and Moczydlowski, 1994; Morabito et al., 1995). This evidence confirmed that STX binds to a single site in the C-lobe of SXPN.

Table 1.5 Comparison of reported biochemical and pharmacological parameters of native bullfrog, recombinant saxiphilin and the recombinant C-lobe of the protein. (see (Llewellyn and Moczydlowski, 1994; Morabito et al., 1995))

Parameter[†]	Native SXPN	Recombinant SXPN	Recombinant C-Lobe
Estimated Mass (kDa)	91	91	40
STX Affinity (K_d)	0.16	0.18	0.75
pH dependent binding ($pH_{0.5}$)	5.7	5.4	5.7
Association Rate K_{on} (s^{-1} M^{-1})	8×10^5	1.8×10^6	1.7×10^6
Dissociation Rate K_{off} (s^{-1})	1.4×10^{-4}	1.6×10^{-4}	7×10^{-4}

[†] [^{3}H] STX binding assays were performed at pH 7.4 and 0 °C

Although some aspects relating to the properties of bullfrog SXPN have been described, the physiological function of SXPN remains a mystery. There is good evidence to suggest potential biomedical importance of the protein in terms of clinical treatment of PSP, but further research is required to investigate the ecological significance, molecular and biochemical interactions of the protein in the whole organism. As a unique member of the TF super-family, SXPN may aid in the elucidation of new functions facilitated by this group of proteins and provide a useful tool for the examination of PST's in biological systems.

1.10 Thesis Aims and Objectives

Clearly at least three distinct classes of STXBPs are capable of specifically binding STX. Of these, only two are soluble plasma proteins (SXPN, PSTBP) and only one is unable to bind TTX (SXPN). The functional and biochemical differences between these three protein groups (see Table 1.6) may distinguish these three STXBP sub-types and thereby facilitate the tentative classification of soluble STXBPs from soluble extracts from a variety of animals.

Table 1.6 Comparative biological and pharmacological parameters of known saxitoxin binding proteins.

Protein	VGSC	PSTBP	SXPN
Species†	human, rat, frog, puffer-fish, crab, insect, mollusc, bacteria	*Fugu pardalis* (puffer-fish)	*Rana catesbeiana* (bullfrog)
Tissue	nerve, muscle, heart	plasma	plasma
Size (kDa)	300	104	91
Subunits	Up to 3 (α, β1, β2)	1	1
Membrane bound	yes	no	no
Biological function	Na^+ ion channel	unknown	unknown
K_d (nM) STX	0.43	8.5	0.16
K_d (nM) TTX	1.8	12000	None
Primary homology‡	K^+ Ion channel	TTXBP	Transferrin
Other Activity	Binds variety of different toxins	Unknown	Inhibition of papain, human cathepsin B and cathepsin L
References*	(Catterall, 1980; Catterall, 1986; Denac et al., 2000b; Goldin, 1999; Satin et al., 1994)	(Yotsu-Yamashita et al., 2002; Yotsu-Yamashita et al., 2001)	(Llewellyn and Moczydlowski, 1994; Mahar et al., 1991; Morabito and Moczydlowski, 1995)

† Selected species are noted for VGSC due to extended phylogenetic range and only species confirmed by molecular techniques are listed for SXPN.
‡ Amino acid sequence homology based on BLASTX analysis
* References from which data was derived

Some marine and amphibian species appear to have developed a number of ways to avoid the toxic consequences of PSTs and associated poisons. For instance some crabs and molluscs which accumulate extreme levels of PSTs (e.g. *A. floridus*) have STX resistant nerves which protect them from the lethal effects of the toxins (Daigo et al., 1988; Kvitek and Beitler, 1991). It is possible that nerve resistance, which involve minor amino acid substitutions of the VGSC (Noda et al., 1989), facilitates the accumulation of high toxin levels, with the biological advantage being distaste and death to predators. Several species of frogs which have STX-sensitive nerves (Kao and Fuhrman, 1967; Strichartz, 1984a) have also been observed to be less sensitive to the toxic effects of STX than mammals (Mahar et al., 1991). These observations have since been associated with circulating levels of the soluble STXBP, SXPN (Mahar et al., 1991). In both

instances, this circumstantial evidence appears to correlate to native environmental conditions which the animals may encounter during their life span. Benthic marine crabs and bivalves are likely to be exposed to dinoflagellate sources of PSTs such as *A. catenella*, and frogs may be exposed to the toxins following contamination of freshwater with cyanobacterial PST sources such as *A. circinalis*.

Despite the ease of these assumptions, there is very little data to support a relationship between soluble STXBPs and PST exposure. In fact, soluble STX binding activity has been observed in marine species that have never been found to accumulate toxins (e.g. damsel and cardinal fish), and in terrestrial arthropods such as centipedes and desert-dwelling reptiles (Llewellyn et al., 1997) that are unlikely to be exposed to substantial freshwater sources of STX. In addition, it is not known whether the STX binding activity that has been observed in soluble extracts from a variety of species has any relationship to known STXBPs such as SXPN and PSTBPs.

Pharmacological evidence suggests that STX binding identified in a variety of amphibians, reptiles, fish and arthropods resembles SXPN based on the lack of TTX binding and similarity of binding kinetics (Llewellyn, 1997; Llewellyn et al., 1997). In many instances it was not clear whether the STX binding activity reported in these studies was proteinaceaous in nature and a preliminary screening study indicated that the detection methods used are inherently unreliable in the presence of biological extracts and salts (see Chapter 2).

These anomalies need to be resolved and proteins fully characterised in order to assign tentative functional classifications as sub-types of STXBPs. Likewise, in order to conclusively classify these proteins, amino acid sequence is required which would unambiguously determine the true relationships between protein groups.

The biological role of VGSC has been extensively studied for many years and while the crystal structure of the protein is yet to be fully elucidated it is clear that the membrane bound protein functions as a Na^+ channel, a critical component of

excitable tissue and nerve conduction (Barchi et al., 1987; Catterall, 1986; Catterall et al., 1986; Denac et al., 2000b; Messner and Catterall, 1986; Moczydlowski and Latorre, 1983). In contrast, the biological role of soluble STXBPs remains a mystery but their potential application in both medical and research applications of PST treatment and detection is substantial.

It is conceivable that a detoxification mechanism exists in animals exposed to PSTs, and the only common function of STXBPs includes the ability to bind STX. Therefore, the main objective of this thesis was to examine various aspects of the biological relationship between STX and soluble STXBPs and the null hypothesis to be examined was as follows:

Soluble, STX-specific binding proteins act as a defence mechanism against STX and its derivatives

The primary objectives of this multidisciplinary research were to:

- Develop a robust assay for the sensitive detection and characterisation of novel STXBPs.
- Extend the known phylogenetic diversity of STXBPs and to characterise the binding and selectivity to STX analogues and TTX.
- Tentatively classify STXBPs based on biochemical and pharmacological parameters.
- Investigate the relationship between protein and ligand with particular interest on the occurrence and distribution of PSTs at known 'Hot Spots'
- Identify new sources and vectors of PSTs in a benthic food web, thus consolidating pathways of STX biomagnification and extending the known diversity of these toxins in the marine environment.
- Select a model for further exploration of the lifecycle and tissue distribution of STXBPs and determine any seasonal variation in STXBP levels.
- Determine whether a native endogenous ligand for STXBPs exists in a model source of the protein.

- Purify and partially sequence a STXBP from a model organism to clarify the molecular relationship of the isolated protein to facilitate future molecular initiatives.

Each objective to be examined closely interrelates (see Fig. 1.7) to provide a cohesive study at different levels of investigation including the development of detection methods (Chapter 2) which then facilitated examination of environmental and phylogenetic diversity in addition to pharmacological activity of novel STXBPs (Chapter 3). A direct investigation of STXBPs at a site of high PST prevalence followed and provided a setting for the examination of the co-occurrence and relationship of protein and ligand in nature (Chapter 4). A comprehensive biological analysis of STXBPs in an animal model, *Bufo marinus,* then facilitated the exploration of molecular relationships of known STXBPs (Chapter 5).

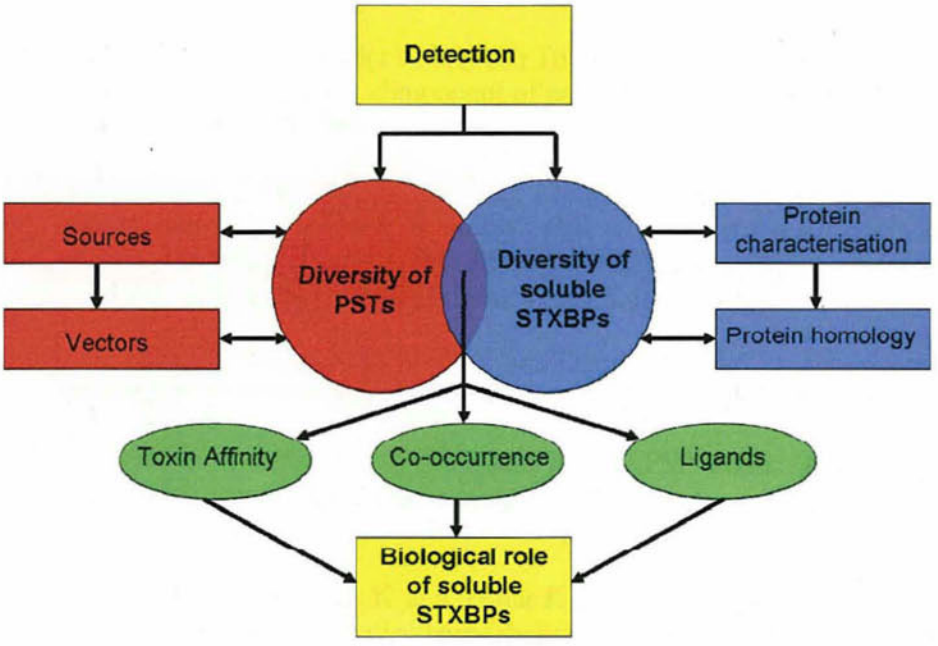


Figure 1.7 Schematic outline of the thesis research plan and relationships of research objectives.

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CHAPTER 2

Development of a Universal Receptor Assay for the Detection of Soluble Saxitoxin Binding Proteins

2.1 Introduction

Saxitoxin (STX) and its derivatives, known collectively as paralytic shellfish toxins (PSTs), represent some of the most potent environmental toxins known. STX blocks voltage gated sodium channels (VGSC) by interaction with binding site 1 on the extra-cellular surface of the alpha subunit (see Fig. 1.3; Chapter 1). The high affinity and rapidly reversible binding of STX, blocks the inward sodium current and subsequent generation of action potentials, rendering them non-functional (Denac et al., 2000). Human STX intoxication is characterised by the rapid onset of physiological symptoms such as tingling of the face, tongue and lips, numbness, vertigo, ataxia, paralysis, and at high doses, respiratory failure and death (Stommel and Watters, 2004).

In the marine environment, PSTs accumulate in shellfish such as bivalve and gastropod molluscs who feed on toxic dinoflagellates from the genera *Alexandrium* (Anderson et al., 1990; Chen and Chou, 1998; Oshima et al., 1992), *Pyrodinium* (Cortes-Altamirano et al., 1995; Mata et al., 1990) or *Gymnodinium* (Anderson et al., 1989; Oshima et al., 1987). In freshwater, PSTs are produced by cyanobacteria including *Anabaena circinalis* (Humpage et al., 1993; Negri and Jones, 1995), *Aphanizomenon flos-aquae* (Mahmood and Carmichael, 1986; Pereira et al., 2000), *Lyngbya wollei* (Carmichael et al., 1997; Onodera et al., 1997), *Cylindrospermopsis raciborskii* (Lagos et al., 1999). Blooms of cyanobacteria can contaminate waterways causing losses in agricultural livestock, affect recreational activities such as fishing and swimming, and may compromise the safety of drinking water (Hoeger et al., 2004; Negri et al., 1995; Orr et al., 2004).

The increased incidence of bloom-forming phytoplankton (Brett, 2003; van Dolah, 2000) has caused aquaculture and wild catch shellfish fisheries worldwide to be more vigilant with their monitoring programs to ensure the release of safe produce to consumers. Likewise, it has become increasingly important to safeguard drinking water for human consumption (Hoeger et al., 2004; Orr et al., 2004) and inform communities of potential hazards of toxic blooms in waterways.

The official testing method for PSTs is the mouse bioassay whereby standardised extracts of sub-sampled shellfish or water are injected into mice and timed to point of last breath to determine toxicity (AOAC-International, 2000), a costly, cruel and non-specific measure. In terms of water analysis this technique has obvious disadvantages, lacking both sensitivity of detection required to assess dilute samples and versatility, where most affected waterways are located in regional areas. Other methods such as analytical HPLC (Oshima, 1995), mass spectrometry (Quilliam, 2003; Quilliam et al., 1993; Quilliam et al., 1989), cell culture (Jellett et al., 1992; Jellett et al., 2002) and radio-receptor assays (Doucette et al., 1997; Llewellyn et al., 1998) have been investigated but most are either not commercially available, require specialist skills and equipment or lack sensitivity. The discovery and characterization of new saxitoxin binding proteins (STXBPs) exhibiting differential selectivity for STX derivatives and/or tetrodotoxin (TTX) may enable the development of sensitive, user-friendly and portable tools for the rapid detection and monitoring of PSP in seafood and water.

2.1.1 Detection and Discovery of STXBPs

Few detection methods for STXBPs have been described to date, and the discovery of novel STXBPs is still in its infancy. The first isolation of soluble STX binding proteins stemmed from observations of high affinity [^{3}H] STX sites in the heart extracts of frogs (Doyle et al., 1982; Tanaka et al., 1984) which led to further observations in the skeletal muscle of the bullfrog, *Rana catesbeiana* (Moczydlowski et al., 1988). A series of pharmacology and biochemical data was accumulated (Mahar et al., 1991) and the protein responsible for the observed STX binding, saxiphilin (SXPN), was purified (Li and Moczydlowski, 1991) and sequenced (Morabito and Moczydlowski, 1994; Morabito and Moczydlowski, 1995). Bullfrog SXPN is a soluble protein approximately 91 kDa which is

homologous to the transferrin (TF) family of iron (Fe^{3+}) transport proteins (Li et al., 1993; Llewellyn and Moczydlowski, 1994) however, lacks the ability to bind either iron or TTX (Moczydlowski et al., 1988). Bullfrog SXPN was characterised using a modified protocol of Ikawa et al. (1982), for the detection of PSTs, using AG50W-X2 cationic resin (Mahar et al., 1991; Moczydlowski et al., 1988). This assay, known as the Dowex assay, relies upon the cationic displacement of H^+ ions from the ion exchange resin by the free radioligand from pre-equilibrated reactions of protein and radioligand (Fig. 2.1).

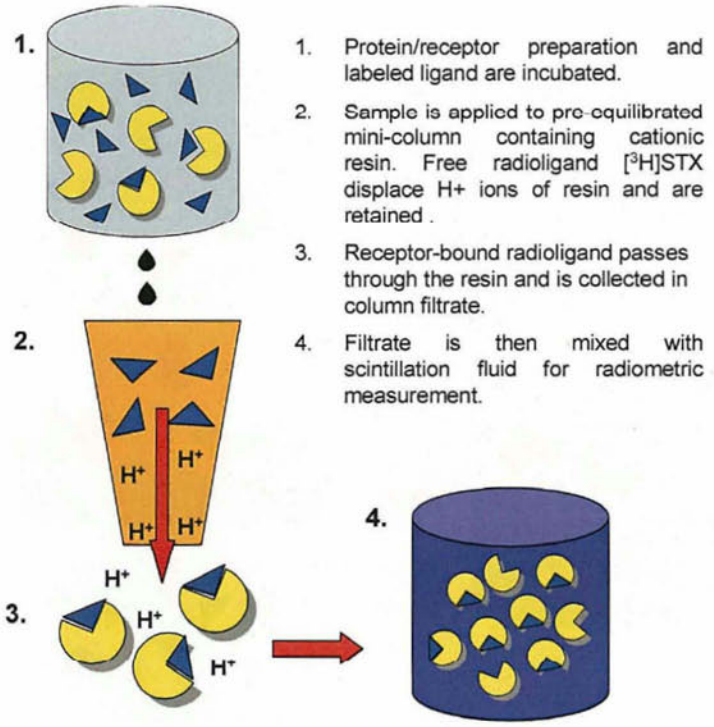

Figure 2.1. Schematic diagram depicting separation of protein bound and free radioligand using cationic exchange resin AG50W-X2 (Dowex).

STX binding activity in the common Australian centipede *Ethmostigmus rubripes,* identified by Llewellyn et al. (1997) was utilised in the development of a screening assay for the detection of PSTs in shellfish extracts (Llewellyn and Doyle, 2001; Llewellyn et al., 1998). This assay relies upon pre-equilibrated "protein" bound [^{3}H] STX binding to glass fibre type B filters pre-soaked with polyethyleneimine (PEI), while unbound [^{3}H] STX is filtered through the

membrane (Fig. 2.2). This method, which has been described as the centipede SXPN assay also incorporates a crude extract from *E. rubripes*. It should be noted however that the relationship of centipede STX binding activity to bullfrog saxiphilin is yet to be elucidated and at this stage there is little evidence to even confirm that a protein is responsible for the activity observed. However, this method has proved to be a useful tool for the sensitive detection and accurate quantitation of STXs (Llewellyn et al., 2002; Negri and Llewellyn, 1998a; Robertson et al., 2004) and a revised format may be a good candidate for the detection and examination of STXBPs in future studies.

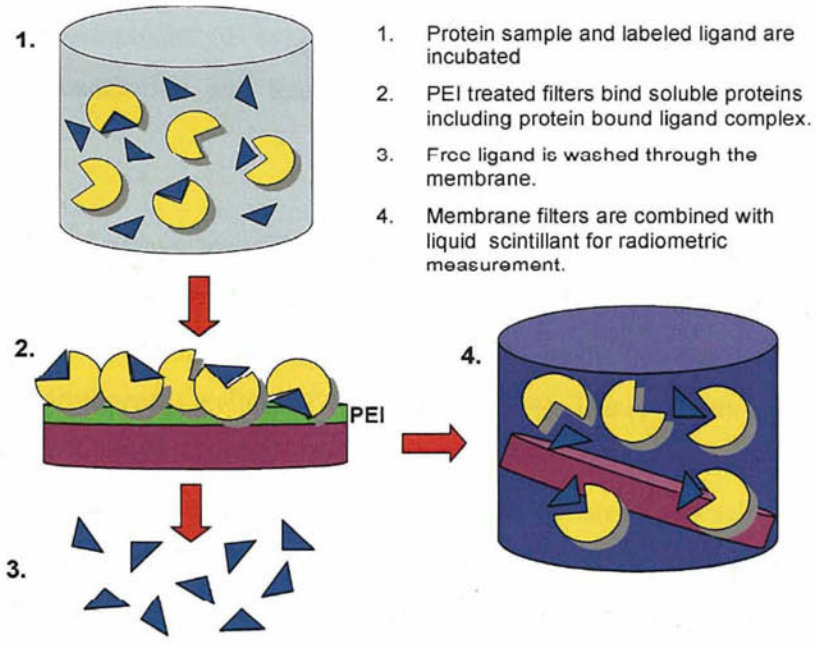

Figure 2.2. Schematic diagram of the separation of protein bound and free radioligand in centipede saxiphilin assay on polyethylimine pre-treated glass fibre type B filters (PEI- GF/B).

2.1.2 Receptor Assays (RA)

Receptors are proteins, anchored in the cell membrane (e.g. VGSCs) or solubilised in the cytoplasm (e.g. TFs), that specifically bind to endogenous neurotransmitters, hormones, and other messenger molecules to exert biochemical responses in cells. Membrane bound receptors may act directly by opening ion channels in the cell membrane that are part of the same receptor molecule, or indirectly by activating second messenger systems that cascade to affect various

processes in the cell. Soluble receptors such as transferrins can transport ligands to other locations within the organism via the bloodstream or directly through membranes by various mechanisms including endocytosis (Descamps et al., 1996). Many drugs, toxins and other naturally occurring compounds can bind to receptors and mimic or antagonise the effects of endogenous ligands. In fact, these interactions can, and have been exploited by scientists to characterise receptors on many occasions, for example, the use of STX and TTX in VGSC research which is well documented (Fozzard and Lipkind, 1996; Lipkind and Fozzard, 1994). Purified ligands derived from endogenous or exogenous sources, become highly useful when labelled with fluorescent or radiometric markers. These labels facilitate development of assays that target specific receptors allowing the detection, quantitation and tracking of the ligand in biological systems and bioassays.

Receptor-based assays are widely exploited in the discovery of natural products for drug development, pesticide and herbicide targets, combinatorial chemistry, pathology and in the monitoring of heavy metals, pesticides and toxins in food and the environment (Devlin 1997; Tan et al., 2004). The receptors used in these highly specific assays can be obtained directly from animal sources (e.g. brain, liver) or expressed in insect or mammalian cell cultures (Litwack 1990). The advantages of receptor assays lie in their ability to detect very small concentrations of ligands in bio-fluids and crude extracts of organisms and when designed appropriately are highly specific to the receptor under investigation. All compounds with the same pharmacological activity can be examined by the same assay. In instances where multiple ligands are present within the same extracts, such as multiple PST derivatives in contaminated shellfish, additional analytical techniques such as high-performance liquid chromatography (HPLC), would be required to differentiate and purify individual toxin derivatives.

2.1.3 Receptor Binding Theory

In basic receptor assays, a labelled ligand of known structure, activity and concentration, usually radio-labelled tritium [^{3}H] or iodine [I^{125}], will compete with an analyte/extract and be allowed to bind the receptor to equilibrium. Following equilibration, the receptor bound and unbound fractions are separated

by filtration, centrifugation or chromatographic methods. Bound and unbound labelled ligand can then be detected according to the properties of the ligand's label e.g. fluorescence detection or radiometric scintillation counting.

Saturation binding experiments are used to determine receptor number and affinity. In these experiments, increasing concentrations of labelled ligand are incubated to equilibrium with a fixed amount of receptor. This interaction can be described by the law of mass action as follows:

$$\text{Ligand} + \text{Receptor} \leftrightarrow \text{Ligand} \bullet \text{Receptor} \qquad \text{(Equation 2.1)}$$

The equilibrium dissociation constant (K_d) describes the affinity of a particular ligand for a receptor and the maximal number of binding sites present in the receptor preparation is defined by B_{MAX}. These parameters can be determined by fitting data (see Fig. 2.3) and are calculated from the following equation where [L*] is the concentration of labelled ligand:

$$\text{Specific Binding} = \frac{[L^*] \times B_{MAX}}{[L^*] + K_d} \qquad \text{(Equation 2.2)}$$

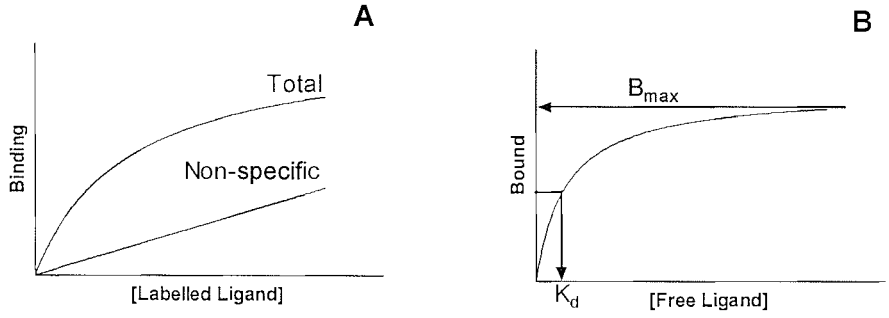

Figure 2.3. Representative saturation binding curves. An increase in concentration of labelled ligand causes a hyperbolic increase in total binding and a linear increase in non-specific binding (A). Extraction of the specific binding (B), allows the calculation of available binding sites (B_{MAX}) and equilibrium dissociation constant (K_d) by linear regression.

A competitive binding experiment measures the binding of a single concentration of labelled ligand in the presence of increasing concentrations of unlabelled ligand. The unlabelled ligand competes with the labelled ligand for binding sites on the receptor. Increasing unlabelled ligand concentrations in the assay will cause a decrease in the amount of receptor bound-labelled ligand. These interactions are described by the equation:

$$[L] + [L^{\pi}] + [R] \leftrightarrow (LR) + (L^{\pi}R) \qquad \text{(Equation 2.3)}$$

where [L] is the amount of unlabelled ligand or analyte added and LR the amount of unlabelled ligand bound to the receptor (R). Plotting the bound fraction of the labelled ligand against the logarithmic concentration of the unlabelled ligand yields an inhibition or calibration curve, from which the affinity and concentration of unlabelled ligand is determined (Fig. 2.4).

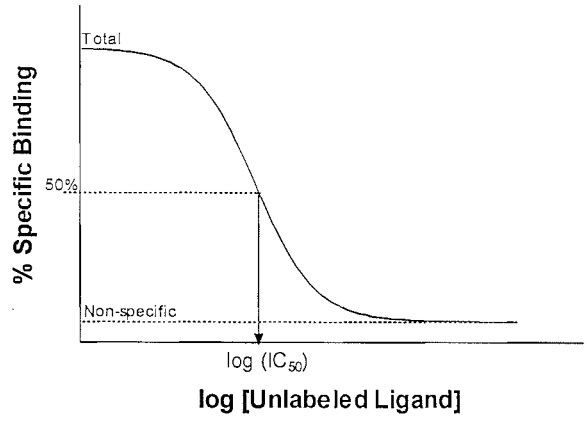

Figure 2.4. Representative competitive binding curve. Increasing concentrations of unlabelled ligand causes a decrease in bound labelled ligand.

Competition binding curves can be described by the following equation:

$$Y = \text{Non-specific} + \frac{(\text{Total} - \text{Non-specific})}{1 + 10^{\log(L) - \log(IC50)}} \qquad \text{(Equation 2.4)}$$

Y is equal to the total binding and log (L) is the logarithm of concentration of unlabelled ligand. The amount of unlabelled ligand which displaces 50% of the bound labelled ligand is known as the IC_{50} or inhibition constant.

This value is inversely proportional to the affinity of the unlabelled ligand for the receptor (K_i) and can be calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973) as follows:

$$IC_{50} = Ki \times \frac{(1 + [L^{\ddagger}])}{K_d} \qquad \text{(Equation 2.5)}$$

2.1.4 Aims of This Study

The discovery of bullfrog SXPN (Mahar et al., 1991; Moczydlowski et al., 1988) and evidence of STX binding activity in some reptile, amphibian, fish and arthropod species (Llewellyn et al., 1997) were facilitated by the use of small AG50W-X2 , H^+ form cation exchange columns (Dowex assay). In these assays, free [^{3}H] STX was separated from receptor bound [^{3}H] STX by filtration, where the charged free [^{3}H] STX was retained by the resin bed after application of aliquots from pre-equilibrated receptor and ligand (Mahar et al., 1991; Moczydlowski et al., 1988). While this method has proved useful in the characterisation and purification of bullfrog SXPN, and has the advantages of solvent and thermal stability, it has several significant drawbacks. Preparation time for this assay involves pre-equilibration and washing of the resin and the tedious task of preparing single-use columns with cotton plug, resin slurry followed by resin bed equilibration and saturation with a blocking solution containing BSA. The assay itself is carried out in alternative tubes or plates with aliquots of each sample then transferred to the columns for individual filtration by syringe propulsion.

In addition to the experimental issues, molecular capture of the radioligand to the resin can be limited by matrix effects such as pH and charge, as was reported following pH titration attempts in newly identified STXBPs . At acidic pH (> 4.0), efficiency of AG50W- X2 resin displacement of [H^+] with [^3H] STX may be reduced causing free [^3H] STX to elute from the resin along with the receptor-ligand complex confounding the accurate detection STX binding activity and causing an apparent false positive. Clearly, as a primary assay for the detection of STX binding activity in a plethora of sample extracts of unknown size, charge and binding characteristics, the Dowex method is problematic and time intensive.

To enhance research output and produce reliable and quantifiable data from novel targets in biological matrices (e.g. tissue extracts, plasma), a primary screening assay must be optimised in terms of preparation time, throughput, cost, specificity and matrix effects. In this study several radio-receptor assays were developed and trialled to identify a suitable primary screening regimen for the detection of STXBPs. Assays utilising charged capture or protein binding surfaces were compared to the Dowex resin based (Mahar et al., 1991; Moczydlowski et al., 1988) and PEI pre-treated GF/B filtration assays (Llewellyn and Doyle, 2001; Llewellyn et al., 1998). Assay conditions were optimised and effects of salt, pH and incubation parameters were investigated to determine the overall technique performance.

The specific aims of this study were to:

- Identify and trial a variety of different radio-receptor assay methods for the detection of soluble STXBPs.
- Develop a primary screening assay for the detection of hydrophilic STXBPs.
- Optimise detection of STXBPs from several confirmed source organisms including *Bufo marinus, Ethmostigmus rubripes, Brachydanio rerio* and *Lophozozymus pictor*.
- Determine whether STX binding characteristics in active extracts follow the assumptions of receptor binding theory.
- Determine the effects of salt, pH and protein loads on assay performance.

2.2 Materials & Methods

2.2.1 Reagents

All buffers and general chemicals were purchased from Sigma (Underwood, QLD), and water was deionised (~18MΩ) with a Millipore MilliQ system (North Ryde, NSW). Ultrafree MC Biomax molecular weight filter units, mixed cellulose syringe filters (0.45μM and 0.2μM), and all 96-well Multiscreen^{TM} microplates (GF/B, HA, PH, DE, IP) were purchased from Millipore (North Ryde, NSW). Norit EurA and Norit GSX activated charcoal was kindly provided by Swift and Co. Ltd. (Nudgee, QLD). Optiphase Supermix scintillation fluid (SF) and 96-well MicroBeta sample plates were supplied by Perkin Elmer (Knoxfield, VIC). Complete protease inhibitor cocktail tablets (protease inhibitors) were purchased from Roche (Dee Why, NSW). Tritiated STX ([^{3}H] STX) was purchased from Amersham Pharmacia Biotech (UK). Unlabelled STX dihydrochloride (STX) was purchased from the National Research Council of Canada (NRC), Institute of Marine Biosciences (IMB) Certified Reference Materials Program (CRMP).

2.2.2 Collection of specimens

Ethmostigmus rubripes (centipede) and *Bufo marinus* (cane toad), were hand collected from urban locations within Townsville, QLD. *To avoid exposure to venom from both of these species a small aquarium net was used and leather gloves worn during collection. Brachydanio rerio* (zebra-fish), *Acheta domestica* (cricket) and *Tenebrio molitor* (meal worm) were purchased from local aquarium suppliers (NQ Pet warehouse & NQ Pet Supplies; Townsville, QLD). *Lophozozymus pictor* (crab) were hand-collected at low tide at locations in the central Great Barrier Reef, QLD. *Penaeus monodon* (tiger prawn) were kindly donated by the AIMS Aquaculture group and plasma from *Bos taurus* (cow) was purchased from Sigma (Underwood, QLD).

2.2.3 Preparation of STXBP Extracts

E. rubripes, B. marinus, B. rerio and *L. pictor* were chosen as positive controls for STX binding activity based on their well described affinity for STX (Llewellyn, 1997). Similarly, extracts of *P. monodon, A. domestica, T. molitor* and *B. taurus* were selected as negative controls based on their lack of STX binding activity in previous studies .

E. rubripes, L. pictor and *P. monodon* specimens were anaesthetised by hypothermia (4°C, 30 min.) and sacrificed. Carapace was removed from the animals prior to rapid freezing in $N_{2\,(l)}$, then tissues were ground to a powder by mortar and pestle. Following reconstitution in 20 mM HEPES-NaOH (pH 7.4) containing a cocktail of protease inhibitors (Roche), extracts were centrifuged for 20 min. at 15000 × g, 4 °C (Hermle Z323K refrigerated centrifuge) then supernatant re-centrifuged an additional two times to remove membrane materials containing sodium channels. Clarified supernatants were filtered through 0.2 μM cellulose acetate filters (Millipore) then stored at -80 °C until use.

B. marinus specimens were humanely killed by pithing, according to the ethical guidelines of the James Cook University Ethics Committee (permit A745-02). Immediately following euthanasia, blood was collected from adult toads by cardiac puncture with Vacutainer™ syringes and lithium coated tubes (BD). Plasma was separated from whole blood by centrifugation in a Beckman GS6 centrifuge at 2500 × g for 15 min. at room temperature (RT). The plasma component was snap frozen in $N_{2(l)}$, freeze-dried, reconstituted in 20 mM HEPES-NaOH (pH 7.4) containing protease inhibitors (Roche) and stored at -80 °C until use.

Whole extracts of *B. rerio, A. domestica* and *T. molitor* were prepared following anaesthesia and euthanasia carried out according to JCU ethical guidelines. Immediately following death, animals were frozen in $N_{2(l)}$ and ground to a powder by mortar and pestle, then reconstituted in 20 mM HEPES-NaOH (pH 7.4) containing protease inhibitors (Roche). Extracts were then filtered through 0.2μM cellulose acetate filters (Millipore) and centrifuged (20 min. 15000 × g, 4 °C; Hermle Z323K refrigerated centrifuge) to remove particulate matter and membrane materials containing sodium channels, and stored at -80 °C. *B. taurus* plasma was reconstituted in 20 mM HEPES-NaOH (pH 7.4) containing protease inhibitor cocktail (Roche) then stored at -80 °C.

2.2.4 Receptor Assay Trial

Seven different radio-ligand assay formats were assessed for their ability to accurately detect and characterise STX binding activity in protein extracts. The performance of the published Dowex radio-receptor assay (Mahar et al., 1991; Moczydlowski et al., 1988) was compared with a modification of the GF/B centipede SXPN STX-binding assay in addition to novel ion exchange filtration assays, protein binding filtration assays and a more traditional charcoal centrifugation assay format. Unless otherwise stated, standard assay conditions comprised of 20 mM HEPES, 1 mM EDTA, 100 mM, NaCl, 2 nM [^{3}H] STX and protein extract and were incubated for 60 min. at RT (~25 °C) to reach steady state conditions. In all cases non-specific binding was defined as the difference in binding measured in the presence and absence of 5 µM unlabelled STX. Background counts were monitored in control samples containing all sample components except for radioligand. Following separation of bound [^{3}H] STX from free [^{3}H] STX, Optiphase Supermix (Wallac) SF was added to bound receptor-ligand bound to filters or aliquots of separated supernatants and thoroughly vortexed for 30 min. at RT. This step ensures that the majority of tritium is released into the scintillant for detection of photons which are released following radioactive decay whereby energy from beta particles is transferred by collision to an electron in the shell of the scintillant (Wang et al., 1992). In all cases counts per minute were measured in a Wallac MicroBeta Liquid Scintillation Counter (Perkin Elmer) at 46% efficiency. Details of each radioligand assay trialled are provided in Table 2.1.

Dowex assay

Protein bound [^{3}H] STX (300 µL) was separated from free [^{3}H] STX by the rapid filtration of 100 µL aliquots of equilibrated assay samples through 1 mL columns of AG50W-X2 resin (100-200 mesh, H^+ form) pre-equilibrated with 20 mM Tris-HCl (pH 7.4), 10 mg/mL bovine serum albumin (BSA; Sigma), followed by a 500 µL ice cold wash (0-4 °C) of 20 mM Tris-HCl (pH 7.4). This cationic resin binds the positively charged [^{3}H] STX and has an approximate molecular weight exclusion limit of 2700, therefore [^{3}H] STX bound by large receptors passes through the column in the void volume (see Fig. 2.1). SF (1 mL) was added to

filtrates and vortexed for 30 min. and then measured by liquid scintillation counting (LSC).

Glass fibre type B filter plate assay (PEI-GF/B)

Described as the GF/B centipede SXPN assay (Fig. 2.2), this method was developed for the sensitive detection of PSTs in shellfish extracts (Llewellyn *et. al.*, 1998; Llewellyn & Doyle, 2001) however for the purposes of this trial, STX binding activity from protein extracts was assessed. Multiscreen^{TM} 96-well GF/B filtration plates (Millipore) were pre-soaked for two hours in 0.3% (w/v) polyethyleneimine (PEI) then aspirated using a vacuum manifold and pump immediately prior to the addition of the assay reagents. 20 mM Mops-NaOH, 200 mM NaCl, 2 nM [^{3}H] STX, and 200 µg protein extract (total protein) were combined to a final volume of 150 µL and allowed to equilibrate for 60 min. at RT. Free [^{3}H] STX was separated from any STXBP-[^{3}H] STX complex by aspiration through the filters followed by three washes with cold deionised water. Filters were air-dried for 10-15 min. then placed into 96-well scintillation counting plates (Perkin Elmer). SF (200 µL) was added to each well, then plates were sealed, vortexed for 30 min. and measured by LSC.

Table 2.1. Comparison of radio-receptor assay methods trialled in the development of a primary screening assay for the detection of STX binding activity.

Assay Name	Assay Format	Separation Method	Filter/Matrix Material	Filter/Matrix Properties
Dowex	Column	Filtration	AG50W-X2 Resin	Cation displacement, 2700 MW exclusion
SXPN-GF/B	Microplate	Filtration	Glass Fibre Type B	Protein binding to polyethyleneimine
PH-cation exchange	Microplate	Filtration	Phosphocellulose	Negative charge
DEAE- anion exchange	Microplate	Filtration	Diethylaminoethyl	Positive charge
MCE- protein binding	Microplate	Filtration	Mixed cellulose esters	High protein binding
PVDF- protein binding	Microplate	Filtration	Polyvinyl difluoride	High protein binding
Charcoal	Microfuge tubes	Centrifugation	Activated Norit EUR & GSX charcoal	Small molecule adsorption

Phosphocellulose cation exchange filtration assay (PH)

200 µL standard reactions containing 15 µg protein extract (total protein) were incubated in 96-well conical plates for 60 min. at RT. Phosphocellulose Multiscreen^{TM} PH plates (Millipore) were pre-wet with 20 mM Tris pH 7.4 for 2-3 min. then aspirated under low vacuum (~3-6 mmHg). 100 µL aliquots of equilibrated samples were then transferred from the incubation plate to the filtration plate and allowed to settle for 10 min. (Fig. 2.5). Free [^{3}H] STX was separated from filters with 3 × 200 µL washes with 20 mM Tris pH 7.4 (0-4 ºC). Finally, PH filters containing bound STXBP-[^{3}H] STX were placed into scintillation plates (Perkin Elmer) and combined with 200 µL SF, then sealed and vortexed prior to LSC.

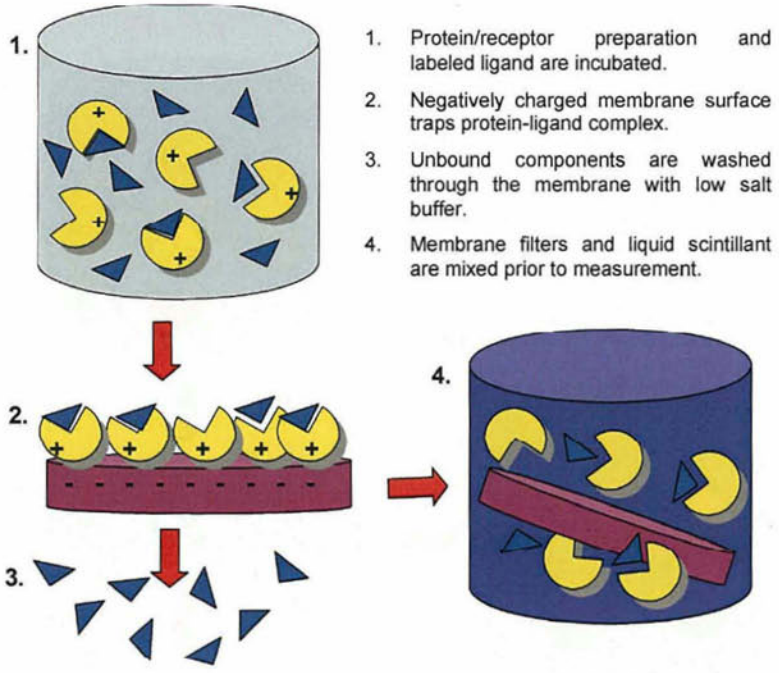

Figure 2.5. Schematic diagram depicting separation of protein bound and free radioligand using a negatively charged cation exchange membrane such as phosphocellulose (PH).

Diethylaminoethyl anion exchange filtration assay (DEAE)

200 µL standard reactions containing 50 µg protein extract (total protein) were incubated in 96-well conical plates for 60 min. at RT. DEAE Multiscreen^{TM} plates (Millipore) were pre-wet with 20 mM Tris pH 7.4 for 2-3 min. then aspirated under low vacuum (4- 6 mmHg). 100 µL aliquots of equilibrated samples were transferred to wells of DEAE filter plates then incubated for a further 15 min. at RT to allow protein binding to the negatively charged DEAE filters. Free [^{3}H] STX was removed by filtration with 3 × 200 µL washes with 20 mM Tris pH 7.4 (0-4 °C; Fig. 2.6). Filters containing bound STXBP-[^{3}H] STX were placed into scintillation counting plates and combined with 200 µL SF, sealed and vortexed prior to LSC.

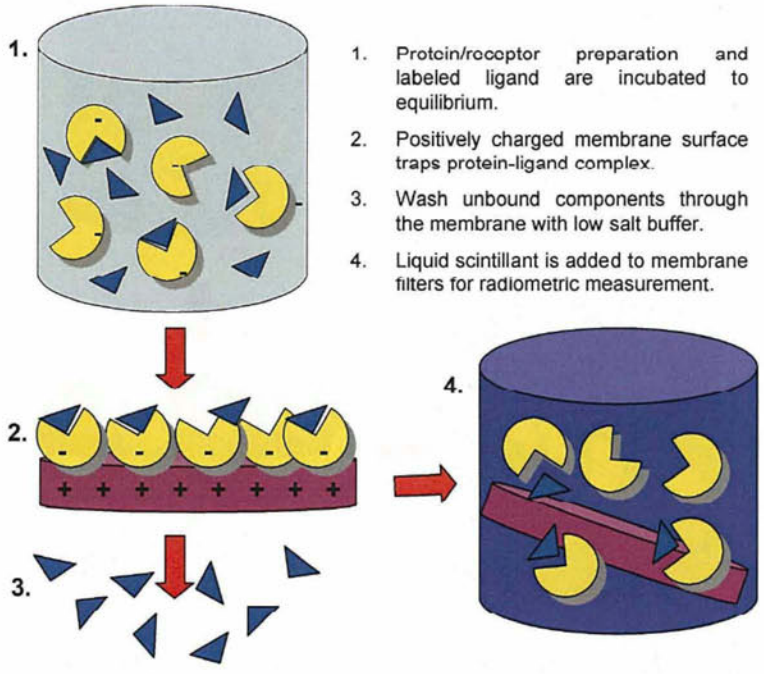

Figure 2.6. Schematic diagram depicting separation of protein bound and free radioligand using a positively charged anion exchange membrane such as diethylaminoethyl (DEAE).

Mixed cellulose ester protein binding assay (MCE)

Protein extracts (20 µg total protein) were assayed by standard conditions in a total volume of 120 µL in a clear 96 well conical plate for 60 min. at RT. Multiscreen™ HA 96 well plates (Millipore) were pre-soaked for 5 min. with assay buffer (20 mM HEPES, 1 mM EDTA, 100 mM NaCl, pH 7.4) then aspirated gently. Following incubation to steady state, 100 µL/ well samples were transferred from the incubation plate to the HA filtration plate then allowed to bind to the filters for 10 min. Free [^{3}H] STX was separated from bound STXBP-[^{3}H] STX complex by vacuum filtration with 3 × 200 µL washes with assay buffer (0-4 °C). Wet HA filters containing bound STXBP-[^{3}H] STX were placed into scintillation counting plates (Perkin Elmer) and combined with 200 µL Optiphase Supermix then sealed and vortexed prior to LSC (Fig. 2.7).

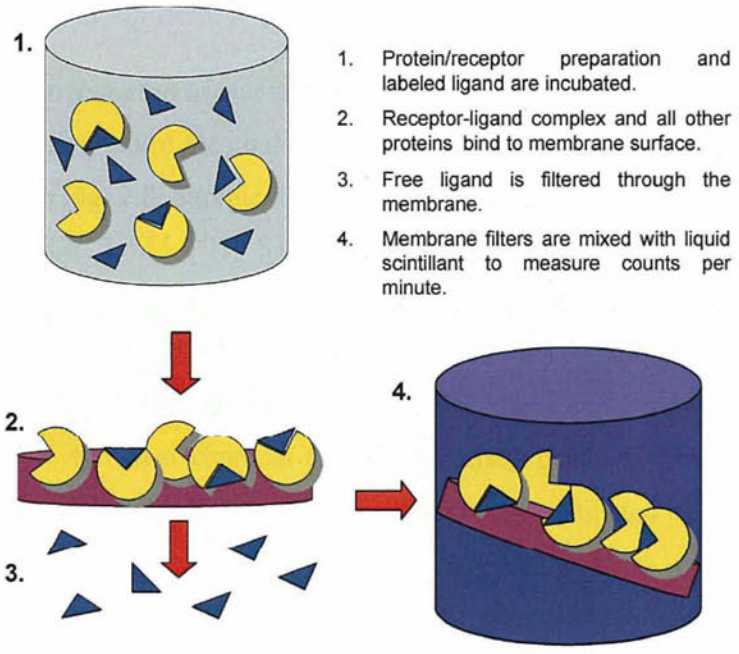

Figure 2.7. Schematic diagram depicting separation of protein bound and free radioligand using high protein binding membranes such as mixed cellulose esters (MCE) and polyvinylidene difluoride (PVDF-IP).

Polyvinylidene difluoride membrane assay (PVDF-IP)

As described for MCE protein binding, protein extracts (20 µg total protein) were assayed by standard conditions in a total volume of 120 µL in a clear 96 well conical plate for 60 min. at RT. Multiscreen™ IP 96 well filter plates (Millipore) were pre-treated for 60 sec. with 100 µL 70% EtOH/ H_20 (v/v), then rinsed with 3 × 200 µL assay buffer (20 mM HEPES, 1 mM EDTA, 100 mM NaCl, pH 7.4) to remove residual ethanol from the wells. 100 µL aliquots of steady state reactions were transferred from incubation plates to IP filter plates and allowed to equilibrate to filters for 10 min. at RT. Free [^{3}H] STX was separated from bound receptor-ligand by filtration with 3 × 200 µL washes cold assay buffer (0-4 °C). Wet immobilin-P filters containing bound STXBP- [^{3}H] STX were placed into scintillation counting plates (Perkin Elmer) for radiometric measurement as described for MCE protein binding assay above (Fig. 2.7).

Charcoal centrifugation assays (CHAR)

A schematic diagram of the charcoal protocol for separating free from bound [^{3}H] STX is shown Fig. 2.8. Sample reactions comprised of 20 mM Tris, 1 mM EDTA, 100 mM NaCl, 2 nM [^{3}H] STX and 100 µg protein extract (total protein), incubated for 60 min. in 1.5 mL microcentrifuge tubes to a final volume of 200 µL at RT. 5 mL stock BSA blocking solution (100 mL) containing 20 g BSA, 0.85 g NaCl and 0.1 g NaN_3, was added to a charcoal slurry containing 5 g Norit GSX or Norit EUR activated charcoal and 45 mL assay buffer (20 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.4). The incubation was terminated by the addition of 200 µL of ice-cold charcoal suspension (10% w/v charcoal, 2% w/v BSA) in Tris-HCl buffer (pH 7.4; 20 mM; 0-4 °C). Samples were then vortexed (5 min.) and centrifuged for 5 min. at 15000 x *g*, 4 °C to separate charcoal-bound free [^{3}H] STX from receptor bound [^{3}H] STX. 100 µL aliquots of the supernatant were transferred to micro-fuge tubes and scintillant added (1 mL). Finally samples were vortexed for 20 min. and measured by LSC.

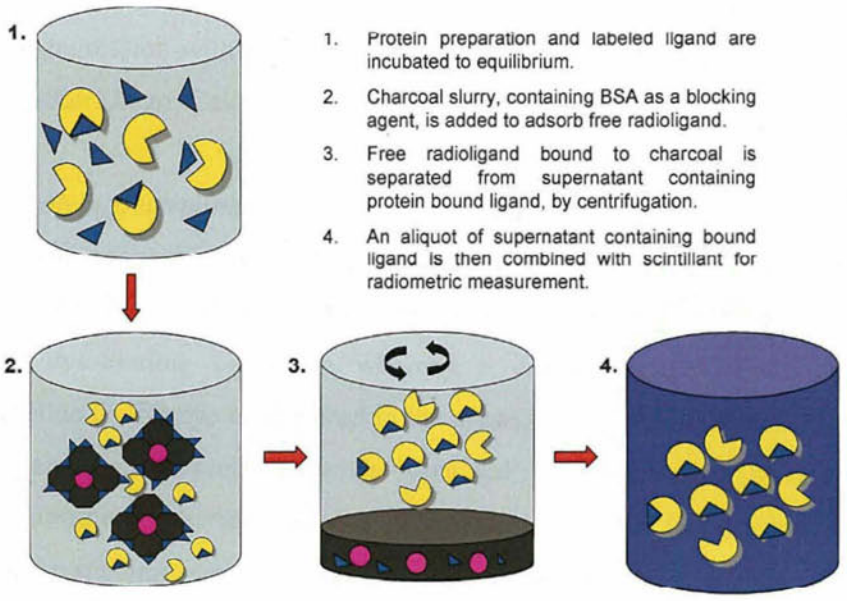

Figure 2.8. Schematic diagram depicting separation of protein bound and free radioligand using activated charcoal and centrifugation (CHAR).

2.2.5 MCE Assay Optimisation

A binding site titration was performed for all positive control extracts to determine the optimal protein concentrations to be used in future screening assays. Crude protein extracts were titrated in the presence of [^{3}H] STX (2 nM). Binding site titration data was fit by non-linear regression with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, California USA). Saturation experiments were performed by using concentrations of [^{3}H] STX in the range 0.1-50 nM with a constant amount of protein as determined from the binding site titrations (50 µg total protein/well). Calibration competition curves were prepared using unlabelled STX while protein content and [^{3}H] STX concentrations remained constant. The effect of increasing salt concentrations on assay performance was determined by varying NaCl and KCl concentrations (0 - 1 M) in otherwise standard assay conditions. In order to determine the effects of pH in the MCE assay, the standard buffer was exchanged for a buffer containing 20 mM Tris; 10 mM MES, 10 mM acetic acid adjusted to desired pH intervals ranging from pH 3 - 9 with either NaOH or HCl, as performed for the centipede micro-titre assay previously described (Llewellyn and Doyle, 2001). Equilibrium binding parameters were evaluated by curve fitting [^{3}H] STX saturation data using

nonlinear regression with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, California USA).

2.2.6 Protein Determination

Total protein concentrations were obtained using the Bio-Rad colourimetric protein assay (Bio-Rad, Reagents Park, NSW). This assay is based on the Bradford dye-binding procedure whereby a colour change of Coommassie Brilliant Blue G-250 dye is observed in the presence of proteins (Bradford, 1976). Colourimetric measurements were obtained at 590 nm (Microsystems Spectrophotometer). Serial dilutions of bovine γ-globulin (IgG) were used to calculate the standard curves.

2.3 Results

2.3.1 Receptor Assay Comparison

Five different methods for the capture and detection of STXBPs were trialled, including cation exchange with positively charged phosphocellulose filter plates (PH), anion exchange using negatively charged diethylaminoethyl filter plates (DEAE), protein binding with filters of mixed cellulose esters (MCE) and hydrophobic polyvinylidene difluoride (PVDF) and charcoal adsorption centrifugation (CHAR) based radio-receptor assays. These five methods were analysed in conjunction with the previously reported AG50W-X2 resin assay (Mahar et al., 1991; Moczydlowski et al., 1988) and PEI-coated GF/B filter plate assay for comparison. *Bufo marinus* (toad) plasma, hemolymph from *Lophozozymus pictor* (crab) and extracts from *Brachydanio rerio* (zebra-fish) and *Ethmostigmus rubripes* (centipede) were used as positive controls based on preliminary data and reports verifying the presence of STX binding activity in these species (Llewellyn, 1997). Extracts and plasma from animals verified as not containing STX binding activity were utilised as negative controls and facilitated the identification of false positive data and included *Penaeus monodon* (prawn), *Acheta domestica* (cricket), *Tenebrio molitor* (meal-worm) and *Bos taurus* (cow).

The performance of each assay is provided for representative species in Fig. 2.9. The Dowex assay effectively detected STX binding activity in active extracts and was reproducible across 5 experiments (Fig. 2.9A). However, non-specific

binding was high and in some instances such as in zebra-fish, non-specific binding was approx 68% of the total binding observed. Also, counts observed in non-active extracts were often lower than non-specific binding results for that species which could lead to the false identification of activity in extracts of unknown activity. In contrast, non-specific binding observed in the PEI-GF/B assay was significantly lower than observed for the same species by the Dowex method (Fig. 2.9B). Centipede STX binding activity was effectively retained on the filters although all other active extracts resulted in low binding efficiency by comparison. Anion exchange separation on DEAE of free [^{3}H] STX from receptor bound [^{3}H] STX was effective in the detection of STX binding activity retained on the filters however all active extracts appeared to have reduced binding efficiency compared to other methods (Fig. 2.9C).

Also in the DEAE method, the non-active *Bos taurus* plasma produced a positive result which was equivalent to that observed for the active centipede extract by the same method and was reproducible across five experiments. Cation exchange on PH filters were clearly unsuitable for the detection of STX binding activity, in most cases producing indistinguishable total and non-specific binding data for each species and data did not correlate to the known activities of each extract (Fig. 2.9D). Both protein binding methods assessed resulted in low non-specific binding and produced accurate and reproducible data across five experiments (Fig. 2.9E-F). The MCE assay produced increased binding efficiency and thus total binding on MCE filters compared to the PVDF and all other methods tested. Finally, the CHAR assays produced lower total binding with high background (Fig. 2.9F).

The performance, reliability and functional aspects of each assay format investigated are summarised in Table 2.2. As preliminary screening studies had indicated, the ion exchange methods were susceptible to variations in extract charge and pH, causing low reproducibility. The charcoal adsorption assay performed moderately with high background the major confounding factor (see Fig. 2.9).

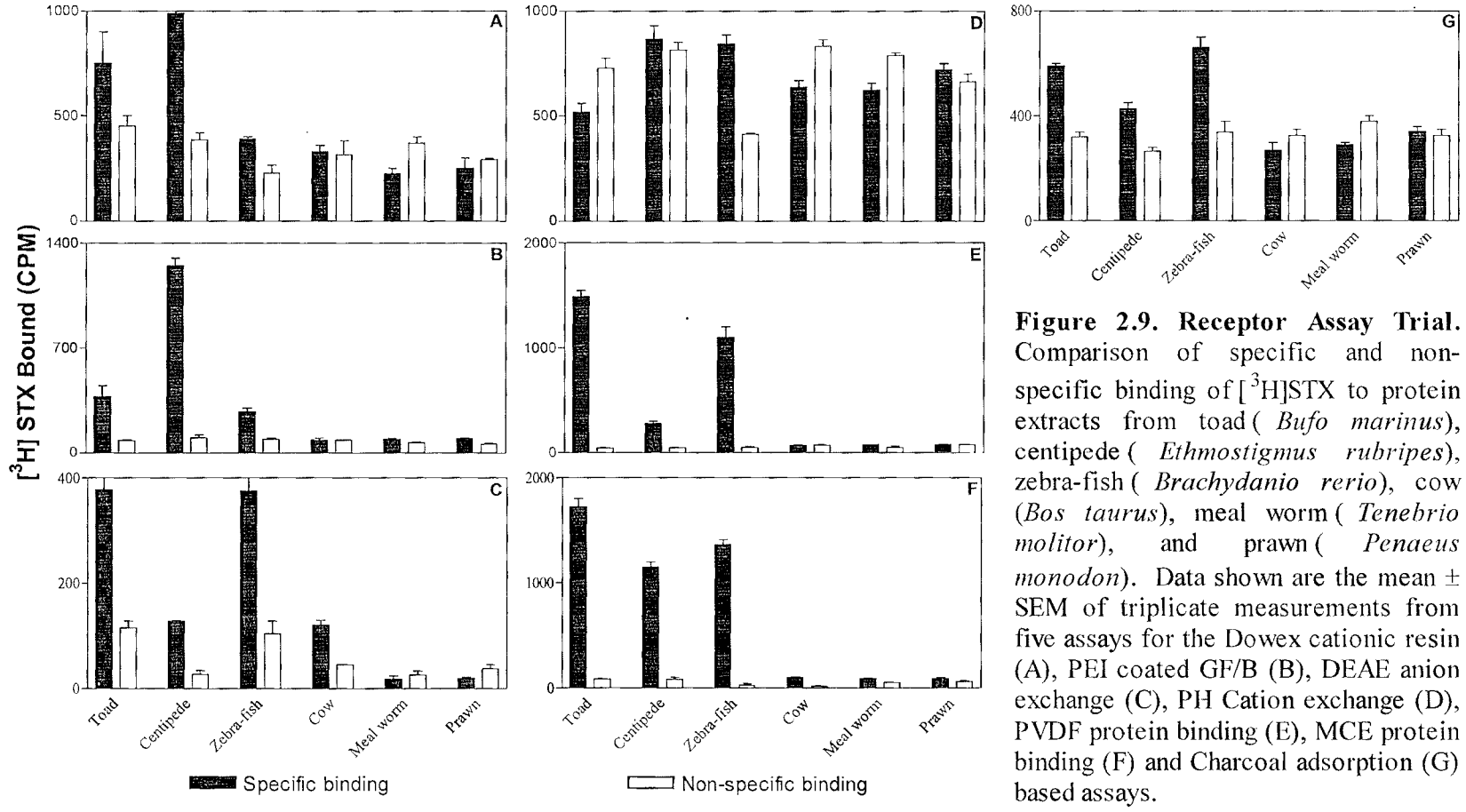

Figure 2.9. Receptor Assay Trial. Comparison of specific and non-specific binding of [^{3}H]STX to protein extracts from toad (*Bufo marinus*), centipede (*Ethmostigmus rubripes*), zebra-fish (*Brachydanio rerio*), cow (*Bos taurus*), meal worm (*Tenebrio molitor*), and prawn (*Penaeus monodon*). Data shown are the mean ± SEM of triplicate measurements from five assays for the Dowex cationic resin (A), PEI coated GF/B (B), DEAE anion exchange (C), PH Cation exchange (D), PVDF protein binding (E), MCE protein binding (F) and Charcoal adsorption (G) based assays.

Table 2.2. Summary of performance of all radio-receptor methods assessed in development of a detection assay for STX binding activity.

Assay Type	Published Assay Formats		Protein Binding		Ion exchange		Traditional
Designation	DOWEX	GF/B	PVDF	MCE	DEAE	PH	CHAR
Matrix	AG 50W Resin	PEI coated glass fiber	Immobilin P	Mixed cellulose esters	Diethyl aminoethyl	Phospho-cellulose	Activated charcoal
% Binding Eff[†]	80	40	60	100	50	60	60
% Background[‡]	25	10	5	5	20	10	30
% Reproducibility[^]	80	80	98	100	50	30	70
Cost/sample*	$ 0.58	$ 0.22	$ 0.32	$ 0.32	$ 0.32	$0.36	$ 0.02
Throughput	L	E	E	E	G	G	G
Handling	L	E	E	E	G	G	G
Matrix effects	pH	pH	Min	Min	Salt, pH	Salt, pH	Min
Solvent Stability	E	G	G	G	G	G	G

E= Excellent performance; G=Good performance; L= Low/ limited performance; Min= minimal effects observed. [†] % Binding Efficiency was determined as a percentage of maximal binding observed in MCE binding assay. [‡] % Background was calculated as the percentage of counts per minute observed in control wells without radioligand compared to total binding for that assay. [^] % Reproducibility was calculated as percentage from 5 consecutive assays of wells producing results within a 95% confidence interval for each replicate. * Cost per sample is given in Australian dollars excluding GST.

The MCE protein binding assay out-performed all other assay strategies with maximal signal observed with MCE membranes, low background, exceptional reproducibility, minimal matrix effects and high throughput. The limiting factor of this assay was protein load which required the determination of protein concentrations in the extracts to allow appropriate extract dilutions prior to use. Based on this data the MCE protein binding assay was selected as the best choice for further scrutiny and optimisation of [^{3}H] STX binding.

2.3.2 Mixed Cellulose Ester Protein Binding Assay

Binding site titrations and saturation binding experiments were performed for all positive control extracts in the MCE protein binding assay. Increasing the amount of total protein in the assay led to a concomitant linear increase in the amount of specifically bound [^{3}H] STX within the range of 1-90 µg protein load (Fig. 2.10). Based on these binding site titrations, a total protein concentration of 50 µg protein/sample was chosen for all standard primary screening assays (Table 2.3).

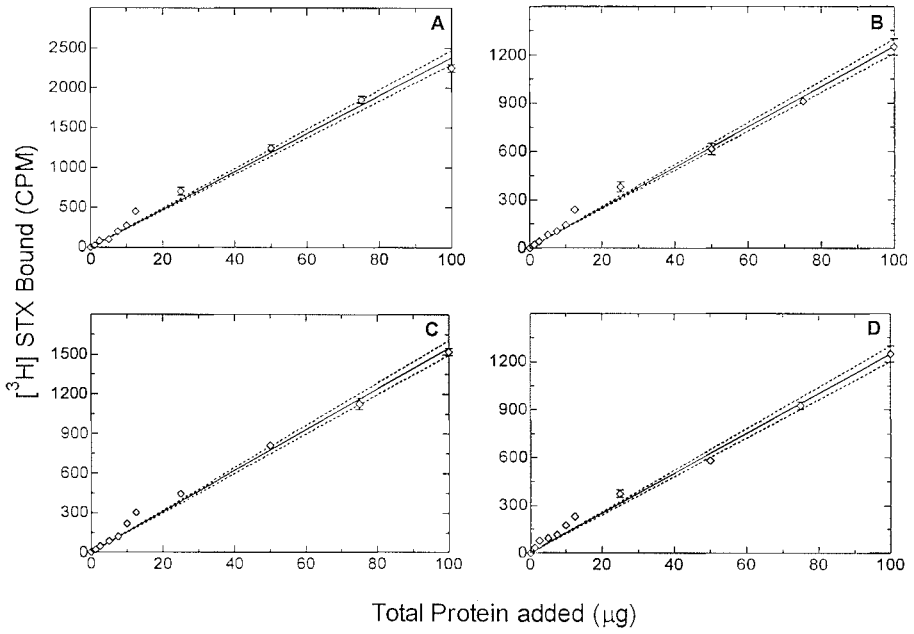

Figure 2.10. Binding site titrations of STXBP extracts from *Bufo marinus* (A), *Ethmostigmus rubripes* (B), *Brachydanio rerio* (C), and *Lophozozymus pictor* (D). Increasing total protein to the MCE STXBP assay caused a linear increase in specifically bound [^{3}H] STX. Data are means ± SEM of triplicate measurements of three assays, in some cases error bars are obscured by the symbols. Dashed lines represent the 95% confidence intervals of the linear regression.

Table 2.3 Optimised assay conditions for the MCE protein binding assay.

ASSAY COMPONENT		OPTIMAL RANGE
Pre-wetting agent		20 mM HEPES-NaOH, pH 7.4
Vacuum		10 mmHg
Incubation temperature		RT (25 °C)
Incubation time		60 min.
Total protein load		10-80 µg
Wash buffer		20 mM HEPES-NaOH, 1 mM EDTA, pH 7.4, 4 °C
No. final washes		3 × 200 µL
Salt range		20- 600 mM
pH range		5 - 9
Assay Components		
	Radioligand	2 nM [^{3}H] STX
	Salt	50 mM NaCl
	Buffer	20 mM HEPES-NaOH; 1 mM EDTA, pH 7.4
	Protein Extract	50 µg (total protein)

Titration of [^{3}H] STX with a constant amount of protein (50 µg protein/ sample) demonstrated that the binding sites were saturable and that non-specific binding was linear (Fig. 2.11). From this data it can be seen that at concentrations between 1-5 nM [^{3}H] STX, non-specific binding was negligible. On this basis a concentration of 2 nM [^{3}H] STX was chosen for use in the standard MCE assay (see Table 2.3).

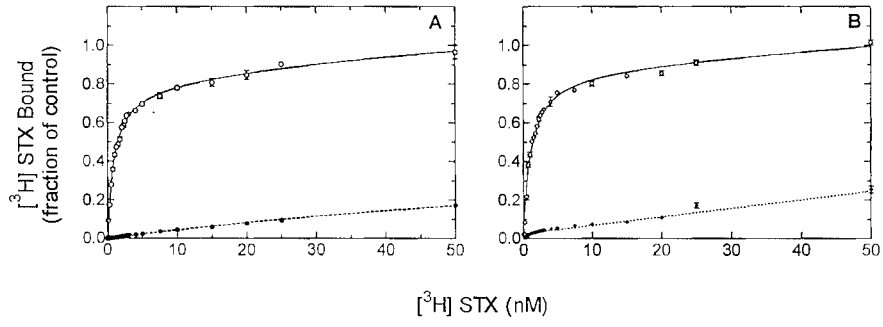

Figure 2.11 [^{3}H] STX saturation binding experiments performed using the MCE STXBP assay. Binding was saturable and non-specific binding was linear and significantly lower than total binding for both *Bufo marinus* plasma (A) and *Brachydanio rerio* extract (B). Total binding is represented by open symbols while closed symbols indicate non-specific binding. Data points are means ± SEM of triplicate measurements from three assays. In some instances symbols obscured the error bars. Binding was saturable and non-specific binding was linear and significantly lower that total binding.

2.3.3 Effects of Salt and pH on Assay Performance

The effect of NaCl and KCl concentration on the MCE assay are illustrated in Fig. 2.12. Assay performance was optimal in the presence of approximately 50-600 mM NaCl and 30-500 mM KCl, in all cases (see Table 2.3). The effect of pH on the MCE assay in the presence of extracts from *B. marinus, E. rubripes,* and *B. rerio* is illustrated in Fig. 2.13. In general, pH between 5 and 9 caused minimal variation in observed [^{3}H] but below pH 5 inhibition of signal was observed.

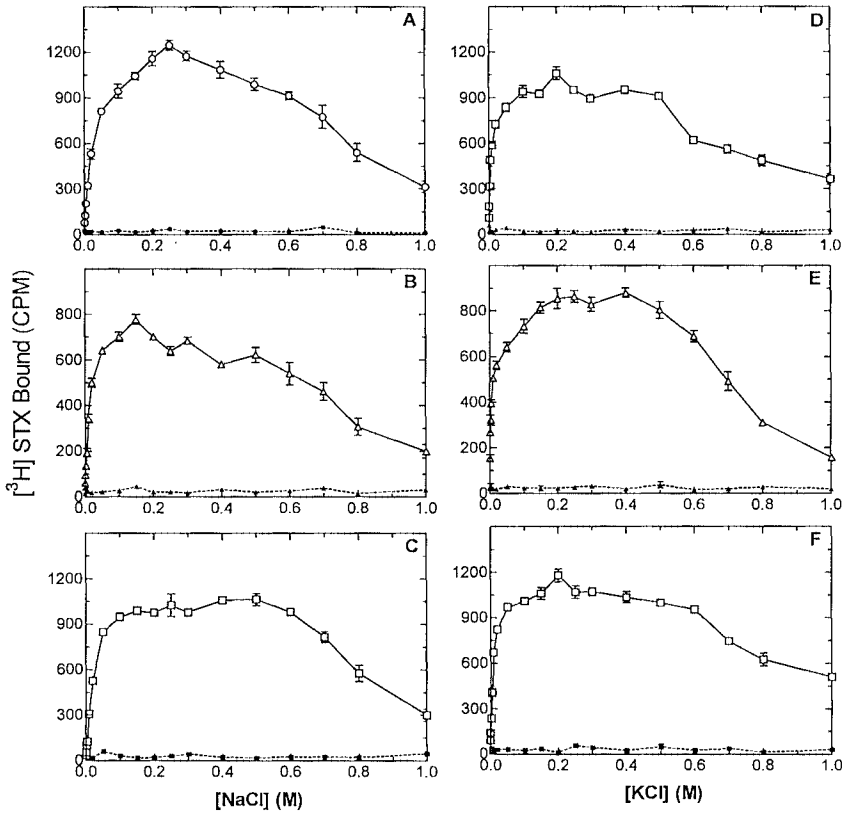

Figure 2.12 Effect of increasing salt concentrations on the performance of the MCE STXBP assay. NaCl (A, B, C) and KCl (D, E, F) were titrated in the presence of 2 nM [^{3}H] STX and 50 mg extract (total protein) for *Bufo marinus* (A,D), *Ethmostigmus rubripes* (B,E) and *Brachydanio rerio* (C, F). Values are the means ± SEM of triplicate measurements from three assays in each case. Open symbols connected with a solid line represent total binding observed while closed symbols connected with a dashed line indicate non-specific binding which was negligible in each case. In some instances error bars are obscured by symbols.

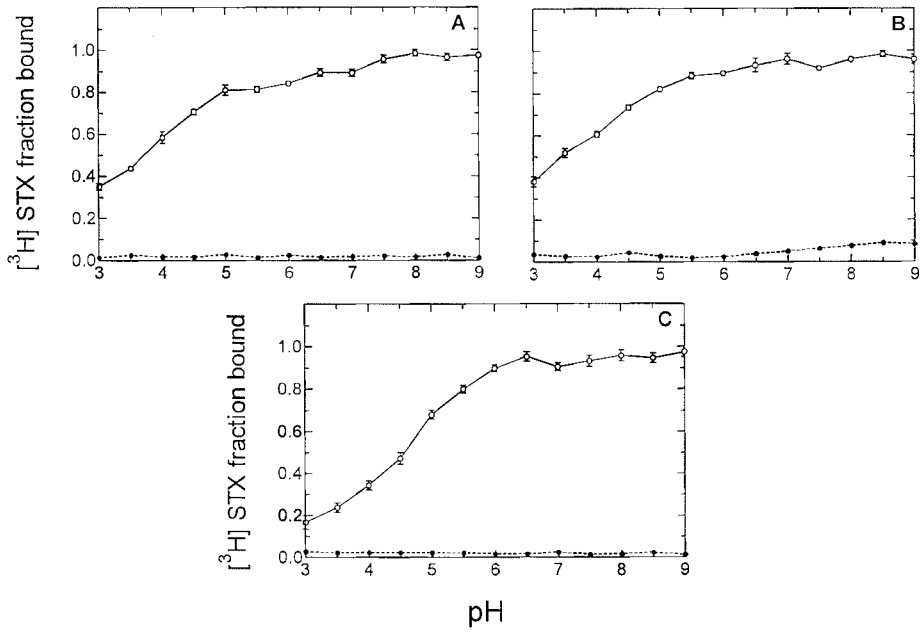

Figure 2.13 pH titrations of equilibrium levels of [^{3}H] STX bound by *Bufo marinus* plasma (A), *Ethmostigmus rubripes* extract (B) and *Brachydanio rerio* extract (C) performed with the MCE STXBP assay. V alues are means ± SEM of triplicate measurements performed in three assays. Open symbols connected by a solid line represent total binding observed while closed symbols with dashed lines represent non-specific binding in the presence of 5 μM unlabelled STX.

2.4 Discussion

2.4.1 Assay Development & Trial

The diversity of soluble STX receptors that exclusively bind STX is known to extend from vertebrate ectothermic species to arthropods (Llewellyn, 1997) but the biological role of these proteins remains a mystery. The discovery and characterisation of new STX binding proteins may provide vital clues to the biological function and aid in the development of sensitive tools for the detection and quantitation of PSTs in shellfish destined for human consumption.

Preliminary screening studies using the Dowex AG50W-X2 cationic resin assay and a modified version of the PEI- GF/B centipede SXPN assay highlighted the need for a more robust method of detecting STX binding activity from biological extracts. Several receptor assays utilising tritiated STX ([^{3}H] STX) were developed, trialled and compared to the Dowex and PEI-GF/B [^{3}H] STX binding assays as summarised in Table 2.2 and Fig. 2.9. Clearly, in instances where all

extract parameters were controlled, the Dowex assay results were consistent and comparable to published data, though with higher non-specific binding than most other methods. However, in screening of crude extracts problems such as high background, chemiluminescence effects of the resin, sensitivity to pH, charge effects and actual handling time, made assaying large numbers of extracts highly problematic and results somewhat ambiguous. These factors combined with the low-throughput, handling and increased cost to run (per sample) confirmed the need to examine other approaches for detection and characterisation of STX binding proteins. It should be noted however that solvent stability was excellent in this assay format which may be critical in future studies of biological function of STXBPs.

The centipede SXPN assay has been well characterised by Llewellyn and co-workers (Llewellyn et al., 2001; Llewellyn et al., 1998; Negri and Llewellyn, 1998b) for (Negri and Llewellyn, 1998a) the detection of PSTs in shellfish extracts and shown to be highly robust to the effects of pH, salt and increasing volumes of shellfish extracts (Llewellyn and Doyle, 2001). However, the binding characteristics of the centipede extract proved to be unique with most other control STXBPs extracts unable to be fully retained on the PEI treated filters. The stronger interaction observed with centipede SXPN could be due to the presence of negative charges on the molecular surface of the protein which has an estimated pI of 5.4 (Llewellyn and Doyle, 2001). The PEI treated filters bear positive charges, so the receptor complex may be retained on the filter by ionic forces during filtration. Apparently, the ionic forces between the filters and STXBP receptors from *B. marinus, B. rerio* and *L. pictor* were not strong enough to retain the receptor material on the filters, which is perplexing given reports of pI values of STX binding activity from these animals also being in the acidic range (Llewellyn, 1997). Ion exchange filter plate methods including the DEAE (anion) and PH (cation) assays were also trialled, with mixed results. Some extracts were able to bind to the DEAE and/or PH filters while others did not, most likely due to slight differences in the charged state of the proteins from different organisms, and negative control extracts often produced false positives, making this method unsuitable for large screening studies. The results observed for the charcoal adsorption method indicated that in addition to the free [^{3}H] STX,

part of the receptor material was adsorbed to the charcoal, despite pre-saturation with albumin. High background counts were also observed in the charcoal trials, indicating that a proportion of free [^{3}H] STX remained in the supernatant subsequent to centrifugation. It appears that the charcoal did not adsorb the free [^{3}H] STX completely which is an acknowledged artifact of this methodology. Wang et al., (1992) described that the reproducibility of the charcoal methods in radioligand binding studies is poor when compared to filtration methods as was observed in this instance.

To reduce the charge and binding effects on separation methods, protein binding matrices were trialled. Millipore Multiscreen™ plates incorporating PVDF-IP and MCE membranes proved to be more reliable and robust than all other methods trialled. These methods bind total protein in the extracts to the filters making separation of free ligand from bound as simple as a series of wash steps, which is also the limiting factor as an overload of protein reduced the effectiveness of binding the protein of interest. A simple protein concentration determination prior to assay resolved this issue and facilitated quantitation of binding sites and specific activity for each STXBP. The MCE protocol produced maximal binding with minimal background, producing reproducible data with no evidence of false positive or negative data elicited by the control extracts. An additional advantage of this method was that the MCE filters dissolved quickly and completely into the SF, unlike all other filtration methods, which would increase the effectiveness of tritium reaction with the scintillant and may also explain the increased count rates that were detected by this method. On this basis the MCE assay was chosen for further study and optimisation.

2.4.2 MCE Assay Optimisation

As mentioned above, protein load to the MCE filters was the only limiting step in the detection of STXBP activity. The incorporation of a protein determination step prior to assay resolved this issue and the assay was effective within a range of approx 1 – 80 µg total protein. Increasing the amount of STXBP extract (total protein) within the protein binding threshold for the filters, caused a concomitant increase in the amount of [^{3}H] STX retained on the filters (see Fig. 2.10). Based on these results a total protein concentration of 50 µg was selected as an optimal

value for analysis of STXBP extracts in future studies. For both *B. marinus* and *E. rubripes* [^{3}H] STX binding was saturable and non-specific binding was linear (Fig. 2.11).

For the centipede SXPN assay to function properly, more than 80 mM NaCl or KCl was required (Llewellyn and Doyle, 2001). In the present study, a reliance on the presence of salt in the assay was also observed, but at concentrations up to 4-fold less that for all STXBPs tested. The greater need for NaCl in the centipede assay could relate to the binding efficiency of the centipede extracts to the GF/B filters rather than a physical requirement in the SXPN-STX binding reaction, which is irrelevant in the MCE protein binding assay. NaCl and KCl concentrations greater than approximately 0.5 to 0.6 M caused an inhibitory effect on the assay for all STXBP extracts, allowing a window of approx 0.5 M in most cases where maximal binding was unaffected by salt concentrations (Fig. 2.12). SXPN-STX binding was not affected between pH 5 and 9 in all STXBP extracts though below pH 5 binding inhibition was observed in all cases (Fig. 2.13). These pH effects are likely to be a product of individual STXBPs pIs for STX binding and it is unlikely to be due to any affect on the ligand used (i.e. STX) as its two pKa values are 8.2 and 11.3 and so the ligand (and hence the radioligand) is not altered by the change in pH in this range.

2.5 Conclusions

This is the first systematic study to develop and trial a range of screening methods for suitability to detect novel STX receptors. Assays utilising charged capture or protein binding surfaces were compared to the standard Dowex and PEI-GF/B filtration assays used in previous studies. In general, the MCE format provided a robust method for the detection of STXBPs and could easily be converted for use as a PST screening tool using *B. marinus* plasma which is readily obtainable worldwide and could be provided freeze dried, as a STXBP source. In Chapter 3, the optimised MCE method was used as a primary screening assay for the discovery and characterisation of soluble STXBPs from biological extracts which facilitated studies on the role of these proteins. In Chapter 4 competitive binding data was assessed to identify differential binding affinities of STXBPs for PST

derivatives, information that could assist in the development of sensitive tools for the isolation and detection of these natural poisons.

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CHAPTER 3

Diversity and Functional Classification of Hydrophilic Saxitoxin Binding Proteins

3.1 Introduction

The most extensively studied soluble saxitoxin (STX) binding protein (STXBP) to date is the transferrin (TF) homolog, saxiphilin (SXPN), isolated from the bullfrog, *Rana catesbeiana* (see Chapters 1 and 2). This protein specifically binds STX (Mahar et al., 1991) and has varied affinity for other paralytic shellfish toxins (PSTs) but does not bind tetrodotoxin (TTX) (Moczydlowski et al., 1988), unlike the membrane-bound voltage gated sodium channels (VGSC) (Denac et al., 2000) and puffer-fish STX and TTX binding proteins (PSTBPs) (Yotsu-Yamashita et al., 2002; Yotsu-Yamashita et al., 2001). At 91 kDa, bullfrog SXPN is considerably smaller than other STXBP sub-types and consists of a single subunit with an internal duplication forming two homologous lobes (Lenarcic et al., 2000). While SXPN has only been purified (Li and Moczydlowski, 1991), sequenced (Morabito and Moczydlowski, 1994; Morabito and Moczydlowski, 1995) and functionally expressed from *Rana catesbeiana* SXPN-like activity has been identified in a variety of species.

One research effort in the field of STXBPs focussed specifically on the phylogenetic distribution of this unique group of proteins (Llewellyn et al., 1997). Extracts of 128 species from 10 phyla were screened for SXPN-like activity (seeTable 3.1). Twenty eight additional species, which included a variety of fish, amphibians, reptiles and arthropods were reported to contain [^{3}H] STX binding activity, and while the proteins were not purified, sequenced or functionally expressed to confirm their sequence or structural homology to SXPN and/or TF, pharmacological characterisation of the protein extracts revealed that a relationship to SXPN was likely (Llewellyn et al., 1997).

Table 3.1 Summary of phylogenetic diversity survey of soluble saxitoxin binding activity reported by Llewellyn *et al.* 1997b.

Phylum	No. Species Tested	No. species Positive
Vertebrata	**56**	**19**
Fish	20	8
Amphibians	6	5
Reptiles	14	6
Birds	5	0
Mammals	11	0
Chordata	**4**	**0**
Echinodermata	**10**	**0**
Annelida	**5**	**0**
Arthropoda	**34**	**9**
Crustaceans	16	6
Insects	16	1
Arachnids	1	1
Uniramians	1	1
Mollusca	**9**	**0**
Total species	**118**	**28**

In addition to SXPN, several soluble proteins have been reported with STX binding activity, notably the PSTBPs, isolated from *Fugu pardalis* (Yotsu-Yamashita et al., 2002; Yotsu-Yamashita et al., 2001). These proteins had no sequence similarity to VGSC or SXPN (see Appendix 1) but their N-terminal sequences were homologous to the partially sequenced TTX binding proteins previously reported in the plasma of *Fugu niphobles* (Matsui et al., 2000). PSTBPs are approximately 106 kDa in size and are single subunit proteins consisting of a non-covalent dimer and can be differentiated from SXPN–like proteins by their high affinity binding to both STX and TTX, and absence of homology to TFs.

Reported STX binding activity from biological extracts can be classified into three distinct functional groups:-

1. Large membrane bound VGSC which are widely distributed throughout the animal kingdom and have dual affinity to TTX and STX.
2. Soluble SXPN receptors which only bind STX and are homologous to TFs.

3. Large, soluble PSTBPs which have dual affinity of STX and TTX but lack homology to both VGSC and SXPN.

Based on these simple functional characteristics, STX binding activity may be tentatively classified into these three groups until such time as individual proteins can be purified, sequenced and functionally expressed.

3.1.1 Aims of this Chapter

Only one STX-specific binding protein has previously been purified, sequenced and expressed (Li and Moczydlowski, 1991; Morabito and Moczydlowski, 1994). While the sequence information gained from this research provided vital evidence regarding homology and activity, the biological role of this unique group of proteins remains a mystery. In this chapter, the diversity of soluble STX-specific receptors was investigated to extend previous phylogenetic surveys and identify any commonality between species that have evolved to express these proteins.

Due to their specific high affinity binding to STX, STXBPs can be utilised in the detection and differentiation of STXs from biological sources, as was exploited in the centipede SXPN radio-receptor assay (Llewellyn et al., 1998). While the centipede assay has been well characterised (Llewellyn and Doyle, 2001) and has facilitated many studies on the occurrence of PSTs from marine and freshwater systems (Llewellyn et al., 2002; Llewellyn et al., 2001; Negri and Llewellyn, 1998), a critical component, the crude centipede extract derived from *Ethmostigmus rubripes*, has limited availability and may be restricted for use in other countries due to quarantine and importation regulations. As such, the assay method in its present form is inaccessible to non-Australian research groups and industries and this is a major drawback in terms of its use as an official method of PST detection. These issues would be eliminated if the protein could be functionally expressed and commercially distributed but in the short to medium term, an alternative and more accessible source of STXBP would be of great benefit. Thus, identification of new sources of STX specific receptors will allow conversion of either the GF/B centipede SXPN assay or a method such as the MCE protein binding radio-receptor assay, which was trialled and developed in Chapter 2 of this thesis, to a more accessible format.

One hypothesis concerning the potential biological functions of STXBPs is that the proteins either serve as a line of defence against STX exposure or (being related to transferrins) facilitate the uptake or transport of PSTs within the organism for some unknown physiological advantage. If either of these hypotheses were true, we would expect to see some level of co-occurrence between STXBPs with PSTs. In this study, all animals collected, extracted and screened for STXBPs were co-extracted and analysed for evidence of STXs to detect any new sources or vectors of PSTs that may explain the evolutionary requirement for STXBPs.

The aims of this chapter were to:

- Collect and screen a diverse range of invertebrates and ectothermic vertebrates for STX binding activity and identify new sources of STXBPs.
- Perform binding site titrations and saturation binding experiments to characterise STX binding in new sources of STXBPs.
- Determine the affinity of selected STXBPs for STX and a variety of derivatives by competition binding with [^{3}H] STX.
- Examine the ability of TTX and Fe^{3+} to compete for STX binding in STXBPs.
- Assess the effects of boiling and trypsin digestion on extract activity and estimate protein size (where applicable) by ultrafiltration.
- Classify new STXBPs into functional STXBP sub-types.
- Identify sources or vectors of PST or TTX derivatives present in the tissues of all collected animals.

3.2 Materials and Methods

3.2.1 Reagents & Standards

All buffers and general chemicals were purchased from Sigma (Underwood, QLD), and water was deionised (~18MΩ) with a Millipore MilliQ system (North Ryde, NSW). Ultrafree MC Biomax molecular weight filter units (MWCO 10 000, 20 000, 50 000, 100 000, 200 000) syringe filters (0.2 µM), and 96-well Multiscreen^{TM} glass fibre type B, glass fibre type C and mixed cellulose ester filter plates were purchased from Millipore (North Ryde, NSW). Optiphase

Supermix scintillation fluid and 96-well MicroBeta sample plates were supplied by Perkin Elmer (Knoxfield, VIC). Cømplete protease inhibitor cocktail tablets (protease inhibitors) were purchased from Roche (Dee Why, NSW).

TTX was purchased from Calbiochem (Kilsyth, VIC) and diluted in 0.05M acetic acid. PST standards including STX dihydrochloride (STX), decarbamoyl STX (dcSTX), neoSTX (neoSTX), gonyautoxins 1,4 (GTX 1,4), gonyautoxins 2,3 (GTX 2,3) and decarbamoyl gonyautoxins 2,3 (dcGTX 2,3) were purchased from the National Research Council of Canada (NRC), Institute of Marine Biosciences (IMB, Halifax, Canada) certified reference materials program (CRMP). Additional PST standards including gonyautoxins 5 and 6 (GTX 5; GTX 6) and C-toxins mixture of 1-4 (C 1-4) were kindly donated by Prof. Y. Oshima and Dr. H. Onodera of Tohoku University, Japan. *Gymnodinium catenatum* toxins 1-3 (GC 1-3) were prepared and supplied by Drs. Andrew Negri and Mike Quilliam as previously reported (Negri et al., 2003).

3.2.2 Collection and Supply of Specimens

Rat brain synaptosomes and *Ethmostigmus rubripes* extract were prepared as previously described (Llewellyn *et al.*, 1998). ZFL cells were obtained from American Type Culture Collection (ATCC) via Cryosite Distribution (Lane Cove, NSW). Cell culture consumables were purchased from Medos (Mt. Waverly, VIC) while media and endothelial growth factor (EGF) were purchased from JRH Biosciences (Parkville, VIC).

194 species were acquired from marine (90 species), terrestrial (76 species) and freshwater (28 species) habitats at locations extending from Bowen to Innisfail in North Queensland (see Fig. 3.1). Nine additional log-dwelling invertebrate species were collected from Tallaganda State Forest, NSW (35° 35'S, 149° 27'E, see Fig. 3.2) with the assistance and under state permits of Dr. David Rowell from the Australian National University (ANU). The majority of animals collected from all sites were invertebrate species from a variety of phyla, with only a few ectothermic vertebrates targeted (see Table 3.2).

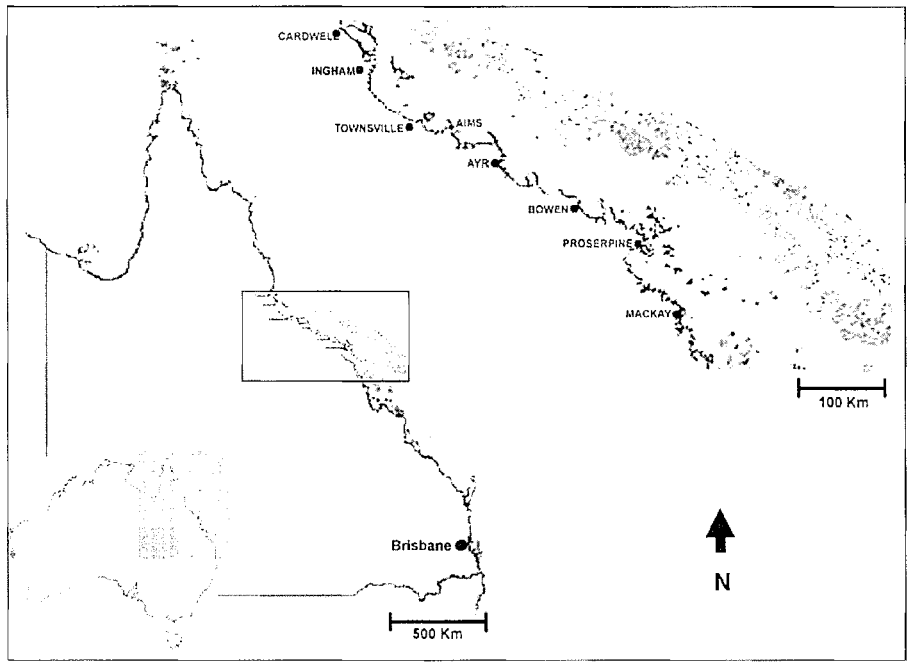

Figure 3.1 Queensland collection sites for the phylogenetic survey of STX binding activity extended from Cardwell to Mackay in marine, freshwater and terrestrial locations.

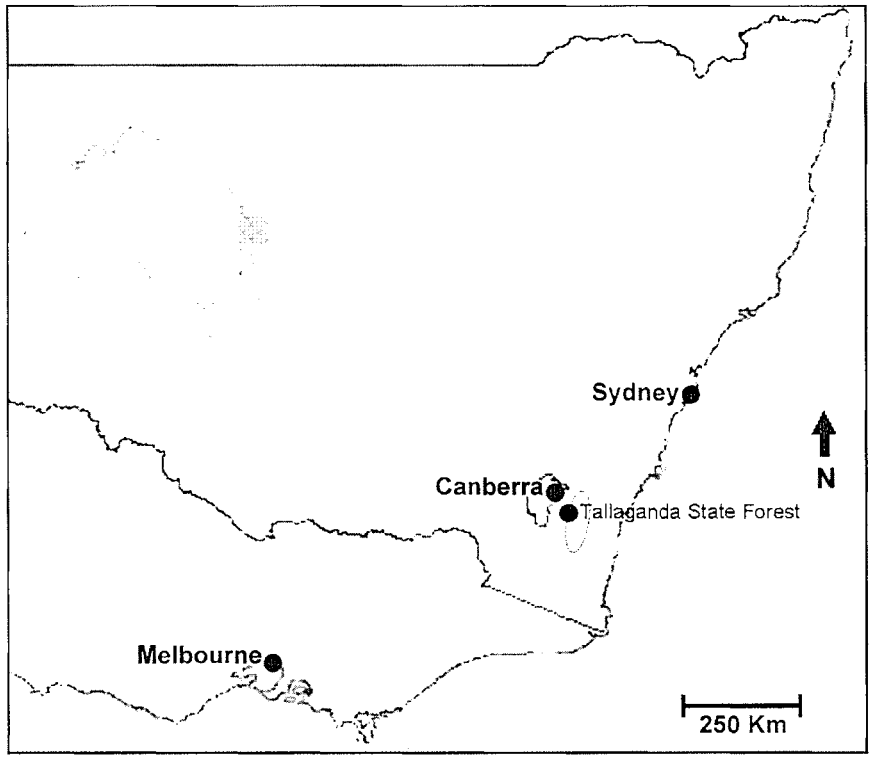

Figure 3.2 Several log-dwelling invertebrates were collected from Tallaganda State Forest (35° 35'S, 149° 27'E), N.S.W.

Table 3.2 Overview of 203 species collected from a variety of QLD and NSW sites and sources within Australia for screening of STX binding activity and PST analyses.[§]

Group	N*	Collection Site/ Suppliers	Identification/ Taxonomy
Porifera	8	GBR	Mr. Carsten Wolfe, AIMS
Cnidaria	16	GBR	Mr. Carsten Wolfe, AIMS
Platyhelminthes	2	NSW	Dr. David Rowell, ANU
Molluscs			
Gastropods	18	GBR	Dr. John Collins, JCU Ms. Alison Robertson, JCU
Bivalves	14	GBR	Dr. John Collins, JCU Ms. Alison Robertson, JCU
Cephalopods	4	Townsville suppliers.	Dr. Mark Norman, MVIC
Annelids	6	Townsville suppliers	N/A
Onychophora	2	NSW	Dr. David Rowell, ANU
Arthropoda			
Crustaceans	18	GBR Townsville suppliers	Dr. David McKinnon, AIMS Dr. Lyndon Llewellyn, AIMS
Insects	40	Townsville, QLD Ingham, QLD	Dr. Simon Robson, JCU Mr. John Hasenpusch, Australian Insect Farm Dr. David Rowell, ANU
Arachnids	16	Townsville, QLD Ingham, QLD NSW	Mr. John Hasenpusch, Australian Insect Farm Dr. David Rowell, ANU
Bryozoans	3	GBR	Mr. Carsten Wolff, AIMS
Echinoderms	6	GBR	Alison Robertson, JCU Mr. Carsten Wolff, AIMS
Ascidians	8	GBR	Mr. Carsten Wolff, AIMS
Vertebrates			
Marine fish	20	GBR Townsville, QLD	Ms. Alison Robertson, JCU Mr. Carsten Wolff, AIMS Ms. Melissa Schubert, JCU
Freshwater fish	15	Aquarium suppliers, Townsville, QLD	N/A
Amphibians	1	Townsville, QLD.	Alison Robertson
Reptiles	6	Venom supplies, S.A.	N/A

[§] See appendix 1 for complete details and numbers of individuals collected.
* Total number of species collected from each category

Marine species were hand-collected from intertidal and exposed reef or by scuba in deeper waters, according to ethical guidelines and marine park permit requirements. A variety of larger marine fish were caught by line fishing with

single hook and kind donations of fresh blood samples from verified species were provided by several local fishermen. Terrestrial species were obtained from hand collections, local aquarium suppliers or the Australian Insect Farm (AIF) located north of Innisfail, QLD.

A variety of reptile and amphibian plasma and venom was provided by Peter Mirtschin from Venom Supplies (VS), Tanunda, SA. Freshwater species were either hand-collected from local rivers and streams, purchased from local aquarium suppliers or were kindly donated by local recreational fishermen. A comprehensive list of all species and sources used in this study is provided in Appendix 2.

3.2.3 Culture, propagation and extraction of Zebra-fish liver (ZFL) cells

Transferrins are primarily produced in liver (Zakin, 1992) and as bullfrog SXPN has been identified as a member of the TFs we would expect that SXPN is likely to also be produced in the liver of organisms in which it is expressed. Evidence from (Llewellyn et al., 1997) indicated that extracts from the zebra-fish *Danio rerio* contained STX binding activity, suggesting the presence of a STXBP. Data collected for this thesis consolidated this evidence with *Brachydanio rerio* extracts revealing saturable STX binding activity (see Chapter 2). Based on this evidence, a cell line derived from the liver of *B. rerio* (Collodi et al., 1994; Ghosh et al., 1994; Miranda et al., 1993), was used to determine whether STX binding activity found in the whole animal had been retained in the development of the immortal cell line.

Zebra-fish liver (ZFL) cells (Cryosite Dist., NSW) were shipped on dry ice to the Cell Culture Facility at AIMS (see Table 3.3). On arrival, frozen cells were quickly thawed then diluted in complete growth media (CGM) containing 50 % Lebovitz's media (L-15; Sigma), 35 % Dulbecco's modified Eagles media with high glucose (DMEM-HG) and 15 % Hams F12 media, supplemented with 0.15 g/L sodium bicarbonate, 15 mM HEPES, 0.01 mg/mL insulin, 50 ng/ mL endothelial growth factor (EGF) and 5 % heat-inactivated foetal bovine serum (HI-FBS), pH 6.8. The cell suspension was immediately centrifuged at 140 × *g* for 10 min. to pellet the cells, then supernatant containing media components and

the cryo-protective agent was discarded. Fresh serum-free media (SFM) was then added and the cell pellet dispersed prior to transfer into a T25 filter capped flask (NUNC) and incubated for 30 min. at 28 °C with free gas exchange with atmospheric air in a humidified environment. After 30 min. cells were examined under an inverted microscope (Olympus) to ensure attachment, 5 % HI-FBS added and flasks incubated as described above until cells were 75-80 % confluent. Spent media was removed and ZFL cells were detached by 2-5 min. incubation at RT (~25°C) with Trypsin-EDTA (0.25 % w/v trypsin; 0.52 mM EDTA). Cells were gently washed in CGM then centrifuged at 140 × *g* for 10 min. to remove trypsin, resuspended in SFM and seeded into NUNC T75 flasks at a density of ~1× 10^6 cells/ mL (split ratio approx. 1:3).

Table 3.3 Summary of ATCC requirements, specifications and characteristics of ZFL cell line from *Brachydanio rerio*.

Designation	*ZFL*
ATCC No.	CRL-2643
Species	*Brachydanio rerio*
Tissue	Normal; Liver
Depositors	D. W. Barnes
Isolation date	1992
Age	Adult
Morphology	Epithelial
Karyotype	Hypodiploid Modal number = 47
Doubling time	72 hours
Growth properties	Adherent
Growth requirements	28 °C; 100 % Air
Known applications	Liver cell metabolism, eco-toxicology, xenobiotic formation.

Cell aliquots were frozen and stored in $N_{2\,(vp)}$ at a density of approximately 2 × 10^6 cells/ mL in CGM containing 5 % DMSO and an additional 10 % HI-FBS. Media was replaced as required, twice per week and cells passaged and seeded every 4-5 days. Prior to extraction, ZFL cells were seeded into 15 mm plates (NUNC) and cultured until 75-80 % confluent. Five random plates were harvested as described, then washed three times in 10 mL of phosphate buffered saline (PBS, pH 7.2) and pelleted by centrifugation at 140 × *g* for 10 min. to generate a single sample.

Triplicate aliquots of sterile SFM, sterile CGM and spent CGM were retained for determination of STX binding. Pellets were drained of remaining PBS and snap frozen in $N_{2\,(l)}$ then treated as described for whole extracts. Five distinct passages of cells were assayed in triplicate to reduce the possibility of a phenotypic anomaly of a single passage.

3.2.4 Sample Preparation

Between three and five individuals of each species collected were extracted to isolate protein and toxin. Prior to extraction, all animals were humanely sacrificed according to the JCU ethical guidelines for the use and handling of animals (Permit No.A745-02). Subsequent to death, all invertebrate individuals were carefully dissected to remove hard shell or carapace, and tissues divided into two sub-samples. Samples were then snap frozen in $N_{2(g)}$ and one sub-sample of each individual was then used for STXBP extraction while the other was reserved for PST extraction as described later. In the case where species size/ weight was very small (e.g. ants, flies) at least 5 randomly selected individuals of a single species were pooled to obtain a single sample for extraction. In these instances at least 3 pooled sampled were prepared ($n \geq 3$). Tissue extracts from whole animals were prepared as described for *E. rubripes* and plasma obtained from larger ectothermic vertebrates was prepared as detailed for *B. marinus* (see Chapter 2). All extracts were centrifuged twice at 15000 × *g* following filtration and prior to assay to isolate the soluble components and eliminate sodium channel contamination.

3.2.5 Primary STXBP screening and characterisation

The optimised MCE protein binding filtration assay, was used for primary screening of protein extracts for STX binding activity. Briefly, duplicate aliquots of standardised protein extracts (50 µg total protein) were incubated at room temperature for 60 min. in the presence of 2 nM [^{3}H] STX, 50 mM NaCl, 1 mM EDTA and 20 mM HEPES-NaOH, pH 7.4 in a final volume of 100 µL. Millipore Multiscreen™ mixed cellulose ester filter plates were pre-wet with 20 mM-HEPES-NaOH, pH 7.4 for 2 min. then aspirated prior to the addition of 100 µL/well pre-equilibrated samples. Samples were allowed to bind to the filters for 5 min. and were then washed 3 times with 20 mM HEPES; 1 mM EDTA, pH 7.4 (4 °C) to remove unbound [^{3}H] STX from receptor bound radioligand. Wet filters

were punched out of filter plates and transferred into scintillation counting plates (Perkin Elmer) where 200 µL Optiphase Supermix scintillant was added. Plates were then sealed and shaken for 30 min. to dissolve filters into the scintillant prior to liquid scintillation counting in a Wallac MicroBeta Liquid scintillation counter (Perkin Elmer) at 46 % count efficiency. Non-specific binding was monitored by incorporation of 5 µM unlabelled STX as previously described (Chapter 2). Any sample exhibiting STX binding at or above 20 % of non-specific binding was considered a positive result. Binding site titrations and saturation binding curves with [^{3}H] STX concentrations ranging from 0.1 to 50 nM were generated for all extracts deemed positive.

Protein quantitation was performed using the BioRad protein assay according to the manufacturer's instructions using immuno-γ globulin (IgG) and bovine serum albumin (BSA) in the construction of standard curves.

3.2.6 Competitive binding experiments

The affinities of the various STX derivatives for protein extracts exhibiting STX binding activity, were determined by their abilities to displace [^{3}H] STX in the MCE protein binding assay previously developed and optimised (see Chapter 2). STX derivatives including STX, dcSTX, neoSTX, GTX5, GTX6, GC-3 and epimeric mixtures of GTX2,3; GTX1,4; dcGTX2,3 and GC 1,2; were assayed three times in duplicate against 2 nM [^{3}H] STX in the presence of pooled protein extracts from any one species. In addition, the affinity of PST derivatives for extracts of acknowledged STXBP sources including the cane toad, *B. marinus* and the zebra-fish, (see Chapter 2) *B. rerio* was assessed. Non-specific binding was determined in the presence of 5 µM unlabelled STX.

3.2.7 Iron (Fe^{3+}) and TTX displacement

The ability of Fe^{3+} and TTX to inhibit [^{3}H] STX binding in all protein extracts identified with STXBP activity was determined by displacement assays and competitive binding experiments. Iron (Fe^{3+}) chloride ($FeCl_3.6H_20$) fragments were dissolved in an acidic solution of nitrilotriacetic acid (NTA) at a ratio of 2.2 NTA/Fe^{3+} adjusted to a pH 4 (NaOH) to produce a stock solution of 10 mM $Fe(NTA)_2$ as previously described (Li et al., 1993). The chelating agent, NTA,

provides a soluble form of Fe^{3+} that can readily bind to available receptor sites as observed for apotransferrin (Harris & Aisen, 1989). Initially high concentrations of $Fe(NTA)_2$ (1 mM) and TTX (500 µM) were incorporated into standard MCE assay conditions as described in section 3.2.4, and reactions allowed to equilibrate for 90 min. In cases where displacement of [^{3}H] STX from the receptor was observed, Fe $(NTA)_2$ or TTX were titrated against 2 nM [^{3}H] STX to determine IC_{50}.

3.2.8 Effects of boiling and trypsin digestion on [^{3}H] STX binding

All protein extracts deemed positive for STX binding activity in previous experiments were investigated further to identify the effect of various physical parameters on observed activity. All positive extracts of the same species were pooled prior to experimentation to increase sample size, and were assayed in duplicate to determine pre-treatment activity. The effect of sample boiling and trypsin digestion were examined to determine whether STX binding activity was derived from a proteinaceous component of the extract. All experiments were performed in duplicate and resultant extracts assayed in duplicate by MCE protein binding [^{3}H] STX assay. Deionised water was adjusted to 100 °C and allowed to boil then duplicate aliquots of extracts/plasma were immersed into the boiling water for a period of 5 min. Samples were then taken out of the beaker and plunged into an ice bath to cool prior to assay. Solution digests of all extracts were prepared using proteomics grade trypsin (Sigma) at a ratio of 1:50 (w/w) enzyme to substrate (extract). Duplicate extracts were dissolved in 100 mM Tris-HCl, pH 8.5, incubated with trypsin at 37 °C for 18 hours, then assayed in duplicate to determine any activity deficit.

3.2.9 Size estimation of STXBPs by ultrafiltration

Assay-guided size estimation of all extracts positively identified with STX binding activity was performed by ultrafiltration with centrifugal molecular weight sieves of various sizes (Microcon; Millipore). 400 µL of protein quantified extract was successively passed through 200 kDa, 100 kDa, 50 kDa, 20 kDa and 10 kDa filtration devices by centrifugation (5000 × g; 60 min. 4 °C). At each step, aliquots of filtrate and retentate were retained for protein quantitation and MCE assay normalised to protein. In addition to newly identified STXBPs, a selection

of previously identified STXBPs were examined including those from *B. marinus* plasma (toad), *L. pictor* hemolymph (crab) and extracts from *B. rerio* (zebra-fish) and *E. rubripes* (centipede).

3.2.10 AOAC PST Extraction

Sub-samples of all animals collected and described in section 3.2.1 of this chapter were co-extracted by a modified AOAC extraction procedure for PSTs (AOAC-International, 2000). All animals were thoroughly cleaned in deionised water prior to extraction. Animals containing hard shells or carapace were carefully removed from their shells prior to extraction. Hydrochloric acid (HCL, 0.1 *N*, pH 3) was combined with whole animals or tissues at 5 mL/ g and homogenised in an industrial blender. Sample extracts were then heated to 100 °C, gently boiled for 5 min. and allowed to cool to RT (~25°C). Following re-adjustment of extracts to pH 3, samples were centrifuged at 3000 × g for 10 min. at 4 °C and supernatants were filtered through 0.45 µM nylon filters to remove cellular debris. Filtered extracts were subsequently lyophilised and reconstituted in 0.05 M acetic acid. The final extract was produced by filtration of the reconstituted supernatant through 10,000 MWCO centrifugal filters (Ultrafree, Millipore).

3.2.11 Radio-receptor assays for the detection of PSTs

AOAC extract inhibition of [^{3}H] STX binding to the sodium channels of rat brain was determined as described previously (Doucette et al., 1997; Llewellyn et al., 2002; Negri and Llewellyn, 1998). The standard assay incorporated Multiscreen^{TM} glass fibre type C filter plates (Millipore) and samples comprised 100 mM 3-[N-morpholino] propanesulphonic acid (MOPS), pH 7.4, 100 mM choline chloride, 1 nM [^{3}H] STX and 25 µg rat brain synaptosomes (total protein) in the presence of AOAC extract (10 µL) in a final volume of 150 µL. AOAC extract inhibition of [^{3}H] STX binding to centipede (*E. rubripes*) SXPN was performed and toxin quantitated as recently reported (Robertson et al., 2004).

3.2.12 Liquid chromatography-fluorescence detection (LC-FD) of PSTs

All AOAC sample extracts which competed with [^{3}H] STX in the radio-receptor assays were analysed by LC-FD for the presence of PST derivatives (see Fig 3.3 for schematic). HPLC analyses were performed by separation on a 5 µM, 250 ×

4.6 mm C18 Alltima column (Alltech, Eagles Farm, QLD) with a Waters 600 HPLC and Waters 717plus auto-sampler combined with a Pickering PCX 5100 post-column reactor at a flow rate of 0.8 mL/ min. Fluorescent derivatives of all PST's were monitored at $\lambda_{excitation}$= 330 nm and $\lambda_{emission}$= 390 nm with a Linear LC305 spectrofluometric detector, as previously described (Negri and Llewellyn, 1998; Robertson et al., 2004). GC toxin analogues were analysed as recently reported (Negri et al., 2003). This technique can be compromised by impostor peaks possessing fluorescence properties akin to the PST's (Baker et al., 2003). To eliminate this possibility, all suspect peaks were interrogated using spiking experiments, measurement of emission maxima and detection in the absence of post-column oxidation (Onodera et al., 1997). TTX derivatives were eluted isocratically with 10 mM heptane sulphonic acid, then oxidised post-column in 4 M NaOH and monitored at $\lambda_{excitation}$= 415 nm and $\lambda_{emission}$= 485 nm (Yotsu et al., 1989). HPLC toxin profiles were converted to absolute toxin values by multiplying the concentration of each toxin in an extract by its toxicity relative to STX (Oshima, 1995) and summing to obtain the total extract toxicity in STXeq.

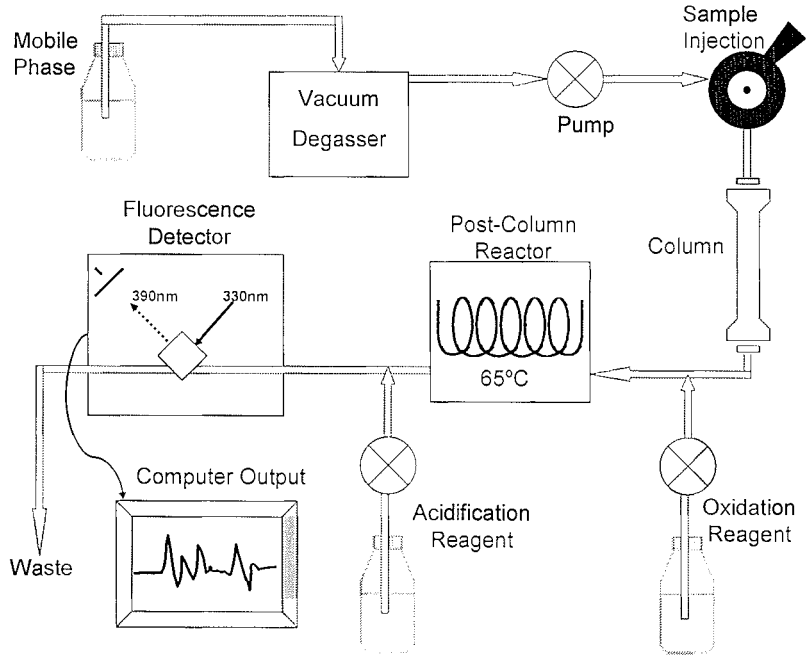

Figure 3.3 Schematic diagram of a high performance liquid chromatography system coupled to a post-column reactor and fluorescence detector as used for the analysis of paralytic shellfish toxins.

3.2.13 MeOH and DCM extracts of STXBP-containing animals

Two sub-samples of approximately 5 g (wet weight) of pooled invertebrates from selected species identified with STX binding activity in addition to tissue from vertebrate species and viscera from *B. marinus* were freeze dried. 10 mL of methanol (MeOH) was added to one sub-sample of each species and 10 mL dichloromethane added to the second dried portion, then homogenised for 1 min using a PRO250 tissue disruptor fitted with a 10 mm toothed head on highest setting. All extracts were then sonicated for 80 min. and allowed to steep for 2 days at room temperature. Extracts were then filtered (0.45 µM nylon, Millipore) and kept at room temperature while remaining tissue was re-extracted an additional 2 times as described, then filtered extracts derived from a single species were pooled and solvent removed by lyophilisation. All extracts were prepared at 10 mg/ mL in specified solvents and assayed in the MCE assay as described in section 3.2.6 to identify any displacement of [^{3}H] STX from respective protein extracts of each species.

3.2.14 Data Analysis

Results are presented as means ± SEM. Equilibrium binding parameters including dissociation constant (K_d), maximal binding capacity (B_{max}) were evaluated by curve fitting [^{3}H] STX saturation or competition data using nonlinear regression with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, California USA). IC_{50} values were converted to K_i using the method of (Cheng and Prusoff, 1973).

3.3 Results

3.3.1 Discovery of new STXBPs

STX binding activity was identified in 8 vertebrate and invertebrate species of the 203 extracted and screened (Table 3.4). In total 32 extracts were active of > 1000 individuals screened. The level of detectable STX binding activity (pmol/g tissue) in these novel STXBP sources ranged from 0.8 pmol/ g tissue in the Antarctic mollusc, *Laternula elliptica* (Fig. 3.4) to 125 pmol/ g tissue in the rainforest scorpion *Liocheles waigiensis*. These studies extended the known phylogenetic diversity of STXBPs to include a species from phylum Onychophora

(*Epiperipatus rowelli*) and Mollusca (*L. elliptica*; Fig. 3.4). Individual variation in STX binding activity was observed in most species, and some STXBP-producing species included individuals without detectable activity (Fig. 3.5).

Extracts of individuals from the same species were pooled for further pharmacological characterisation. Binding site titrations of pooled active extracts revealed that increasing the amount of extract (total protein) in the assay caused a concomitant linear increase in the amount of receptor bound [^{3}H] STX as would be expected in the presence of 1:1 binding of receptor to ligand. Saturation binding experiments revealed that in all active extracts [^{3}H] STX binding was saturable and contained a component of linear non-specific binding (Fig. 3.6). Binding titration data was then analysed with GraphPad Prism to derive equilibrium dissociation constants (K_d) and maximal number of binding sites (B_{max}) for [^{3}H] STX, based on the assumption of a single class of binding sites (see Table 3.5).

Table 3.4 Characterisation of novel saxitoxin binding activity by saturation binding experiments in extracts from a variety of vertebrate and invertebrates. [#]

Species name	Common name	N[†]	Extract Type[‡]	Specific Activity	
				pmol/ gmo tissue	l/ mg protein
Gambusia hamiltoni	Mosquito fish	5	I, T	92 ± 1	19.4
Torquiguener pleurogramma	Toad fish	5	I, T	13.8 ± 0.5	2.9
Ceriodaphnia sp.	Water flea	5*	P, T	2.0 ± 0.5	0.4
Liocheles waigiensis	Rainforest scorpion	5	I, T	126 ± 2	26.5
Lycosa furcillata	Wolf spider	5	I, T	55.6 ± 0.8	11.7
Cormocephalus sp.	Rainforest centipede	5	I, T	68 ± 2	14.5
Epiperipatus rowelli	Velvet worm	5*	P, T	4.1 ± 0.3	0.9
Laternula elliptica	Geoduck	3	I, T	0.8 ± 0.9	0.2

[#] Only species with novel STX binding activity are listed.
[†] N, refers to the number of individuals of a given species that was extracted and assayed.
[‡] I= Individual; P= Pooled; T=Tissue.
* The value of N for pooled animals refers to the number of extracts rather than the number of individuals.

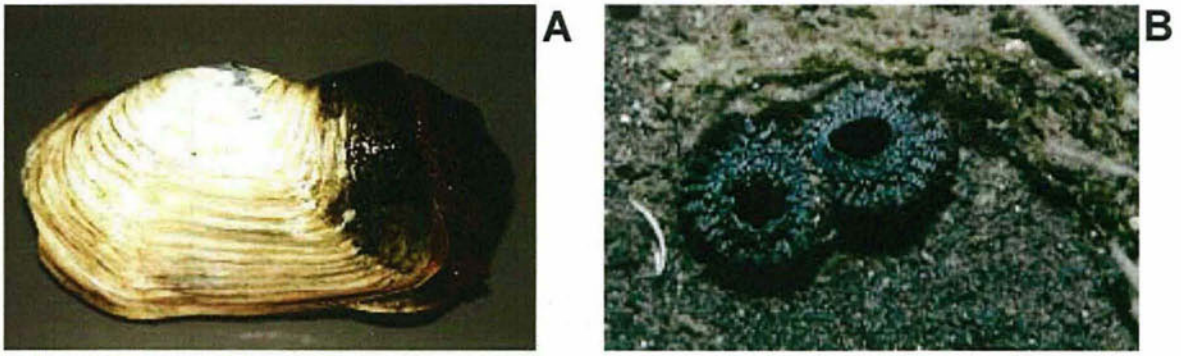

Figure 3.4 Photographs of *Laternula elliptica* depicting whole animal subsequent to collection (A) and prior to collection in Antarctic sediments illustrating siphons (B). Photographs taken and kindly provided by Dr. Andrew Negri.

Table 3.5 Characterisation of novel saxitoxin binding activity by saturation binding experiments in extracts from a variety of vertebrate and invertebrates.[#]

Species[#]	Saturation binding*	
	K_d (nM)	B_{max} (pM)
Gambusia hamiltoni	2.2 ± 0.3	71 ± 3
Torquiguener pleurogramma	14 ± 1	38 ± 2
Ceriodaphnia sp.	3.6 ± 0.3	75 ± 4
Liocheles waigiensis	1.7 ± 0.2	61 ± 2
Lycosa furcillata	12 ± 1	71 ± 11
Cormocephalus sp.	5.3 ± 0.4	56.5 ± 0.9
Epiperipatus rowelli	18 ± 2	42 ± 1
Laternula elliptica	26 ± 2	31 ± 1

[#] Only species with novel STX binding activity are listed.
* Data are means ± SEM from three experiments performed in duplicate derived from curves fit by non-linear regression assuming one site binding with Graphpad Prism.

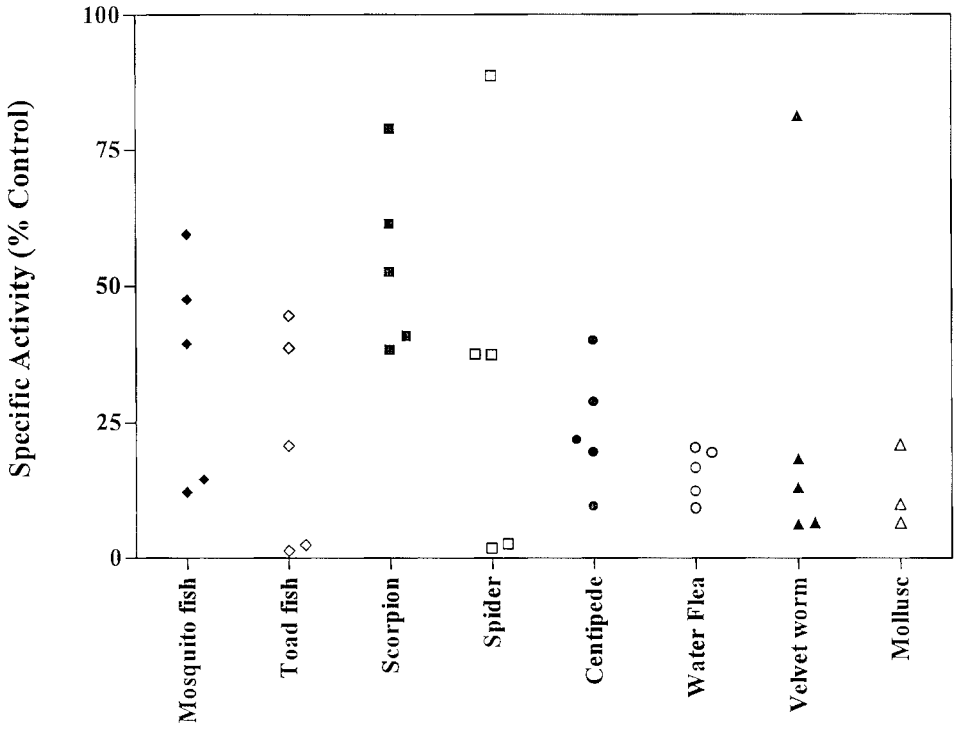

Figure 3.5. Individual variation in specific activity of protein extracts from organisms exhibiting saxitoxin binding activity. These organisms included the mosquito fish *Gambusia hamiltoni*, toad fish *Torquiguener pleurogramma*, scorpion *Liocheles waigiensis*, centipede *Cormocephalus sp.*, water flea *Ceriodaphnia sp.*, velvet worm *Epiperipatus rowelli*, and mollusc *Laternula elliptica*. Data points represent the means of duplicate measurements from three experiments for each individual extract and were standardised to protein and are a percentage of *Bufo marinus* plasma control. All extracts were prepared from individual organisms except in the case of the water flea, *Ceriodaphnia sp.* where extracts were prepared from pooled extracts as described in Methods.

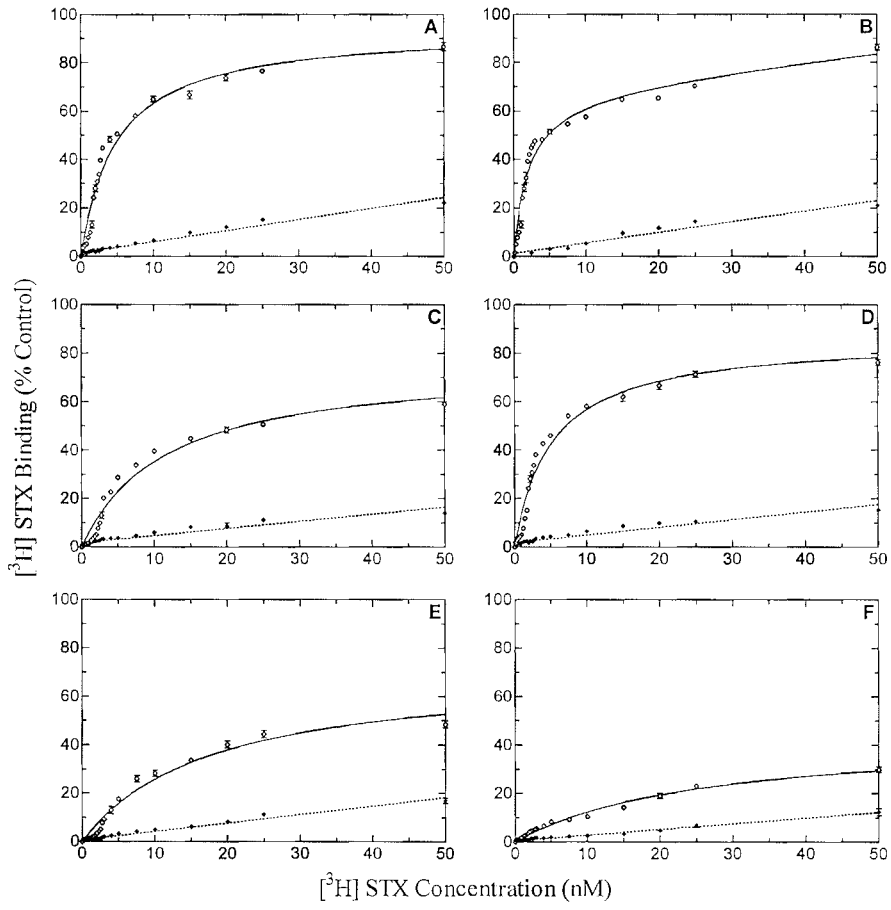

Figure 3.6 Saturation binding of pooled extracts from *Gambusia hamiltoni (A)*, *Liocheles waigiensis (B)*, *Lycosa furcillata (C)*, *Cormocephalus sp. (D)*, *Epiperipatus rowelli (E)* and *Laternula elliptica (F)*. Non-linear regressions of total binding were fitted to a one site model (solid line) and non-specific binding was fitted by linear re gression (dashed line) with GraphPad Prism. Data points are represented as the mean ± SEM from three experiments assayed in duplicate. In some instances error bars are obscured by the symbol.

3.3.2 Zebra-fish Liver (ZFL) Cells

A low level of STX binding activity was observed in extracts of zebra-fish liver cells (ZFL) across five distinct passages of cells and was not detected in any fresh or spent media components (Fig. 3.7). The level of activity was not sufficient to construct full saturation or competitive binding curves but was consistently measured in all three sample extracts prepared for each passage of cells, assayed in triplicate.

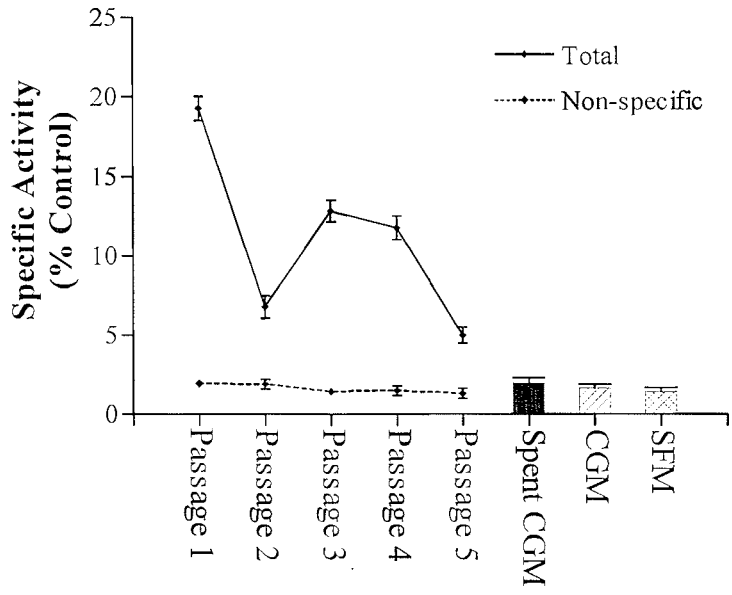

Figure 3.7 Saxitoxin binding activity of media and cell extracts from zebra-fish liver cell (ZFL) from *Brachydanio rerio*. Activity is expressed as a mean ± SEM of activity from Bufo marinus plasma and was standardised to protein in all cases. CGM refers to complete growth media while SFM refers to serum free media. Multiple passages of cells were assessed to reduce possibility of phenotypic anomaly.

3.3.3 Competitive binding Experiments

Competitive binding of ten STX derivatives against 2 nM [^{3}H] STX for extracts from *B. marinus, B. rerio, G. affinis, Ceriodaphnia sp., L. waigiensis, Cormocephalus sp., E. rowelli* and *L. elliptica* was examined (Table 3.7). Extracts from all species tested demonstrated high affinity binding within the same order of magnitude for STX, dcSTX and NeoSTX with the exception of the crustacean *Ceriodaphnia* sp. which showed significantly lower affinity for dcSTX and

NeoSTX (see Table 3.7). In contrast extracts of the mollusc, *L. elliptica* exhibited high affinity for all toxin derivatives lacking R2 or R3 substitution (see Fig. 3.8) including STX, dcSTX, NeoSTX, GTX5, GTX6 and GC-3 (Table 3.7).

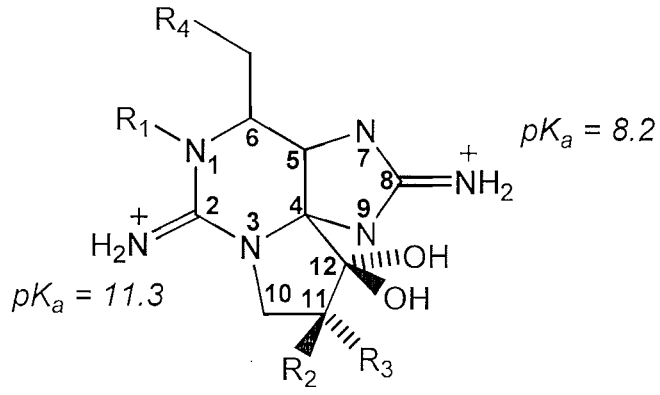

Figure 3.8 Basic structure of saxitoxin highlighting functional groups R_1-R_4 and pK_a of each guanidinium residue.

3.3.4 Iron and TTX

[^{3}H] STX was displaced by 5 μM TTX in displacement assays of the toad fish, *T. pleurogramma* (see Fig. 3.9). Analysis by linear regression with one-site competition revealed an IC_{50} of 55 ± 1 nM for TTX with a Hill-slope of 0.56 ± 0.07. TTX did not inhibit or affect the binding in extracts from any species. The effect of Fe^{3+} was also examined but did not affect protein binding to [^{3}H] STX in any of the extracts tested.

3.3.5 Effects of boiling and trypsin treatment on STXBP activity

All STXBP positive extracts were boiled and digested in trypsin to determine whether the activity was likely to be proteinaceous. In all cased 100 % loss of activity was observed following these treatments.

Table 3.6 Structure-activity relationships between STX derivatives and concentration standardised protein extracts of animals identified with STX binding activity as determined by competitive binding with 2nM [^{3}H] STX in the MCE receptor assay.

Species	K_i (nM)*									
	STX	**dcSTX**	**NeoSTX**	**GTX 5**	**GTX 6**	**GC-3**	**GTX 1,4†**	**GTX 2,3†**	**dcGTX 2,3†**	**GC- 1,2†**
Bufo marinus	2.6 ± 0.1	1.9 ± 0.4	5.6 ± 0.2	22 ± 2	26 ± 1	83 ± 3	14 ± 2	12 ± 2	4.5 ± 0.3	167 ± 8
Brachydanio rerio	2.9 ± 0.1	2.0 ± 0.2	12 ± 1	180 ± 12	224 ± 20	570 ± 34	130 ± 9	156 ± 10	129 ± 7	683 ± 19
Gambusia hamiltoni	3.2 ± 0.3	4.7 ± 0.4	15 ± 2	146 ± 6	200 ± 5	480 ± 19	343 ± 32	290 ± 14	266 ± 20	570 ± 41
Ceriodaphnia sp.	2.8 ± 0.2	431 ± 4	219 ± 3	176 ± 2	190 ± 8	301 ± 4	33 ± 3	19 ± 2	186 ± 13	468 ± 22
Liocheles waigiensis	2.0 ± 0.1	2.8 ± 0.3	4.3 ± 0.5	26 ± 2	29 ± 2	203 ± 8	142 ± 6	114 ± 4	87 ± 2	250 ± 12
Cormocephalus sp.	1.8 ± 0.4	3.5 ± 0.2	6.8 ± 0.6	58 ± 4	70 ± 5	94 ± 3	160 ± 8	138 ± 5	102 ± 4	189 ± 10
Epiperipatus rowelli	3.4 ± 0.5	2.1± 0.4	4.2 ± 0.3	12 ± 1	43 ± 2	438 ± 21	234 ± 11	167 ± 5	118 ± 4	-
Laternula elliptica	2.5 ± 0.2	2.8 ± 0.3	4.1± 0.1	3.4 ± 0.2	2.9 ± 0.3	3.6 ± 0.6	-	-	-	-

* Data are mean ± SEM from 3 experiments performed in duplicate and calculated by one-site homologous competition with GraphPad Prism as described in 'Methods'.
- No inhibition observed therefore K_i could not be determined
† Epimeric mixtures

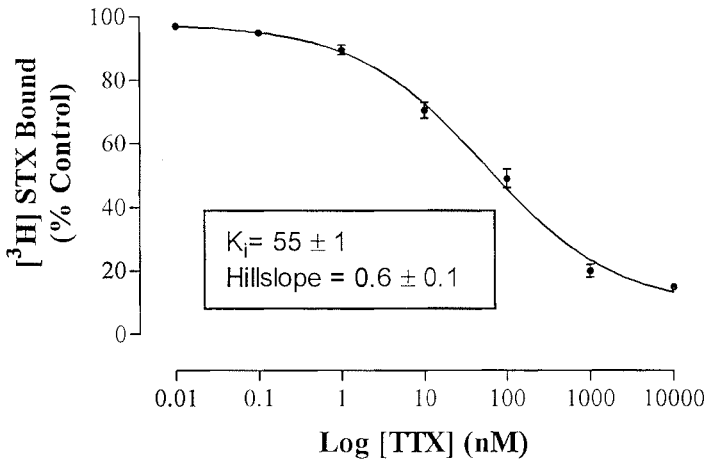

Figure 3.9 Competition binding curve of *Torquiguener pleurogramma* extract showing inhibition of [^{3}H] STX binding by tetrodotoxin (TTX). Data points are the mean ± SEM from three assays performed in duplicate. In some instances error bars are obscured by the symbol.

3.3.6 Size estimation by ultrafiltration

Ultrafiltration of STXBP extracts was performed to gain a gross estimate of the size range associated with STX binding activity (Table 3.7) STX binding activity in the majority of extracts including *Ceriodaphnia sp., L. waigiensis, Cormocephalus sp., E. rowelli, B. marinus, E. rubripes, B. rerio and L. pictor* was recovered at molecular weights between 50 - 100 kDa, with some activity remaining at sizes between 100 – 200 kDa (see Table 3.7). STX binding activity from the toad-fish, *T. pleurogramma* was recovered in fractions greater than 100 kDa while STX binding activity from *L. elliptica* was recovered in filtrates less than 50 kDa (Table 3.7). In all cases a small discrepancy in % activity was observed indicating that some protein was not recovered which may be attributed to protein retained on the membranes of the centrifugal devices.

Table 3.7 Molecular weight based ultrafiltration of pooled extracts of species identified with saxitoxin binding activity.

Species	Ultrafiltration Ranges (KDa)					% Activity Loss‡
	<20	>20 <50	>50 <100	>100 <200	>200	
	STX binding activity observed (% Control) †					
*Gambusia hamiltoni**	-	2.1	71.4	23.7	-	2.8
*Torquiguener pleurogramma**	-	-	-	41.2	52.8	6.0
*Ceriodaphnia sp.**	-	-	89.2	4.1	-	6.7
*Liocheles waigiensis**	-	5.1	61.2	29.0	-	5.7
*Lycosa furcillata**	-	-	13.4	85.1	5.8	-4.3
*Cormocephalus sp.**	-	-	68.8	23.7	-	7.5
*Epiperipatus rowelli**	-	-	76.9	12.3	-	10.8
*Laternula elliptica**	9.5	74	6.4	1.2	-	8.9
Bufo marinus	-	-	93.2	1.9	-	4.9
Ethmostigmus rubripes	-	-	75.8	12.5	-	11.7
Brachydanio rerio	-	-	84.0	9.2	-	6.8
Lophozozymus pictor	-	7.4	68.4	16.2	-	8.0

- Below detection limits
† Data are reported as means obtained from 3 ultrafiltration experiments assayed in duplicate.
‡ Activity loss was measured as a percentage of activity that could not be accounted for or was lost during the course of the experiment due to binding to molecular weight filters or degradation.
* New species identified with STX binding activity during this study.

3.3.7 Occurrence of PSTs in AOAC extracts

To assess the co-occurrence of PSTs with STXBPs and to examine new sources of PSTs, all animals collected for the STXBP screening study were extracted for PSTs according to the AOAC approved protocol. STXeq values from respective assays were calculated from the nM values derived from calibration curves using the molecular weight of STX^{2+} of 301 and the sample dilution factors. All extracts were screened in the sodium channel and SXPN radio-receptor assays and inhibitory extracts were titrated and analysed for PSTs and TTX by LC-FD. Sodium channel assay inhibition was observed in 22 individuals including crustaceans, molluscs, fish and sea urchin (Table 3.8). SXPN assay inhibition was observed in only 17 of these individuals and included the crustaceans and molluscs and low inhibition in two of five toad fish (*T. pleurogramma*) individuals (see Table 3.8). LC-FD analysis of all extracts tested in the radio-receptor assays confirmed the presence of PSTs in 14 individuals (Table 3.8).

Low concentrations of TTX were identified by LC-FD in six extracts including *Lophozozymus pictor* (2), *Liomera tristis* (2) and *T. pleurogramma* (2). The only species analysed whereby the presence of PSTs co-occurred with evidence of STXBP activity was *L. pictor*. Moderate correlations of the PST extracts deemed positive by radio-receptor assays and HPLC analysis were obtained (see Fig. 3.10).

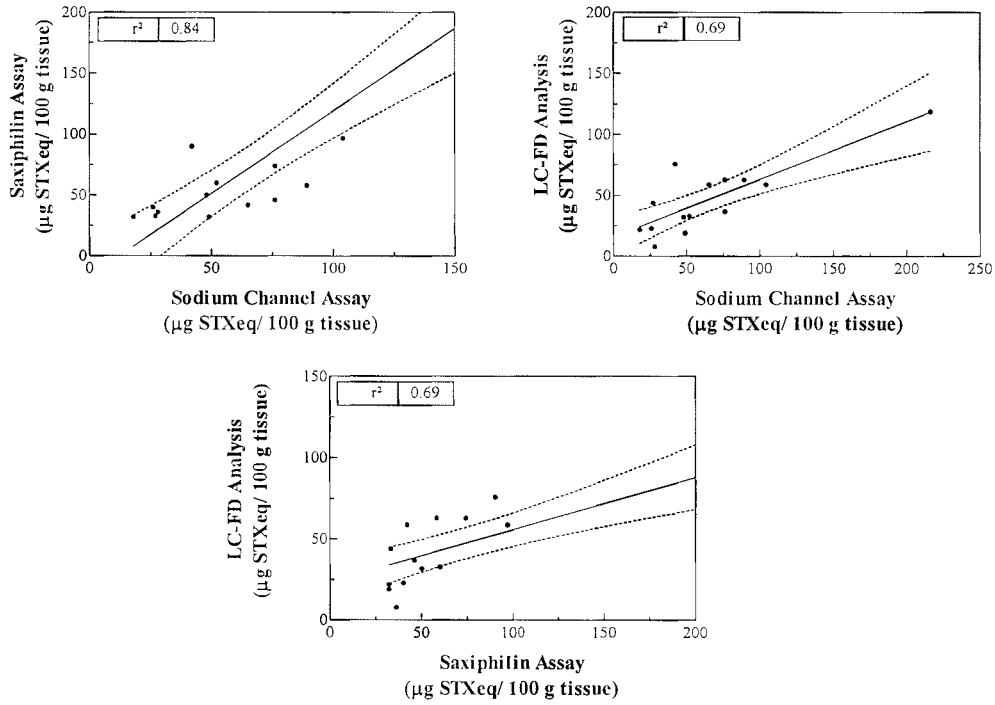

Figure 3.10 Correlations between sodium channel, centipede saxiphilin assay and LC-FD analysis for the calculation of STX equivalents (µg STXeq/ 100 g tissue). Dashed lines represent 95 % confidence intervals of each regression line calculated with GraphPad Prism. Only data confirmed by all three methods were analysed.

Table 3.8 Toxin concentration of individual AOAC extracts defined as toxic by at least one method. Calculation of STX equivalents was performed as previously described (Robertson, *et al.* 2004)

Species	ID	STX equivalents (µg/100g tissue)†			TTX µg/100g tissue
		NaCh-Assay	SXPN-Assay	HPLC	
Atergatis floridus	1	89	58	63	-
	2	104	97	59	34
	3	216	321	119	-
	4	42	90	76	-
	5	18	32	22	-
*Lophozozymus pictor**	6	27	33	44	8
	7	26	40	23	-
	8	49	32	19	2
Liomera tristis	9	76	46	37	12
	10	76	74	63	3
Thalamita crenata.	11	48	50	32	-
	12	52	60	33	-
Tectus pyramis	13	28	36	8	-
	14	23	49	-	-
Turbo argyrostomus	15	65	42	59	-
	16	52	-	-	-
Torquiguener pleurogramma	18	32	9	-	11
	19	28	12	-	8
Diadema sp.	20	116	-	-	-
Acanthopleura sp.	21	101	-	-	-
	22	130	-	-	-

- Below detection limits
* STX binding activity also observed for this species (Chapter 2).
† Mean reported to nearest whole number

3.3.8 Endogenous Ligands of STXBPs

DCM and MeOH extracts of the *Gambusia hamiltoni, Ceriodaphnia sp, Liocheles waigiensis, Cormocephalus sp., Epiperipatus rowelli, Bufo marinus, Brachydanio rerio* were prepared and assayed in the MCE radio-receptor assay to determine whether an endogenous ligand of their individual STXBPs was present. These particular species were chosen based on sufficient supply for extraction and demonstrated STX-specific binding activity. DCM extracts of all species did not inhibit [^{3}H] STX binding to their respective STXBP extracts however a MeOH extract of *B. marinus* viscera partially displaced [^{3}H] STX from diluted plasma collected from the same animal. This activity could not be titrated accurately due to low level inhibition (>20 %, data not shown) but will be examined further in Chapter 5. No further activity was identified in any other MeOH extracts prepared.

3.4 Discussion

3.4.1 Diversity of STXBPs

A diverse collection of invertebrates and several ectothermic vertebrates were gathered from marine, terrestrial and freshwater sources with the expectation of extending the known phylogenetic range of animals expressing STXBPs. Previous screening studies identified 28 new sources of STX binding activity and included a variety of amphibians, reptiles, fish and arthropods (Llewellyn et al., 1997). In this study more than 1000 individuals from 203 different species from the marine, terrestrial and freshwater environments were collected, extracted and screened, extending the range of investigation from previous studies substantially (Fig. 3.11). Of these 203 species, eight were identified with STX binding activity (Table 3.4). These STXBP containing organisms included five arthropods, one fish, one onychophoran species and a mollusc, extending the previously identified diversity of STXBPs (see Fig. 3.12). Of great interest is the presence of STX binding activity in the protein extracts of the temperate onychophoran, *Epiperipatus rowelli* and the Antarctic mollusc, *Laternula elliptica* which extends the known diversity of STXBPs further than previously acknowledged level of arthropods.

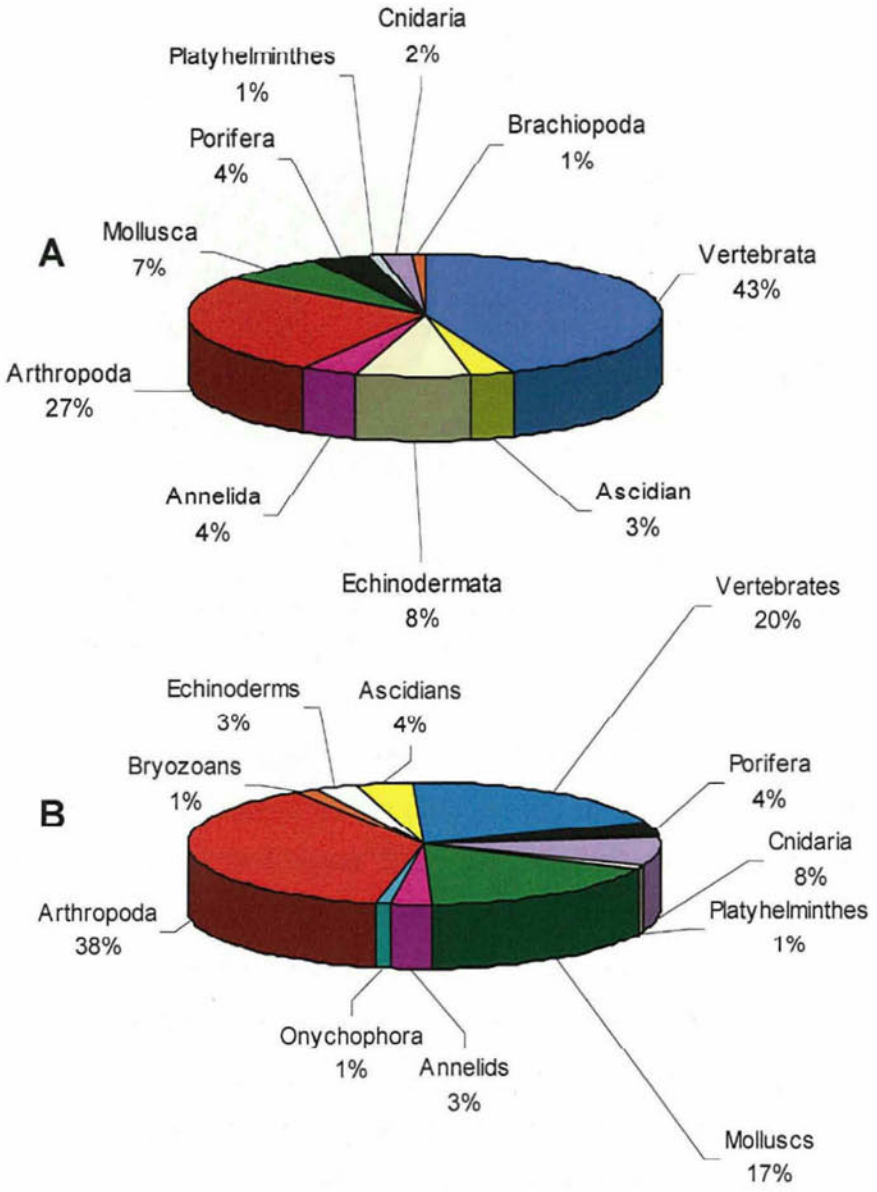

Figure 3.11 Comparison of number of species examined during phylogenetic surveys of saxitoxin binding activity. Data includes a previous screening study by Llewellyn et al, 1997, where 128 species were collected (A), and the present collection in which 203 different species were collected and screened for saxitoxin binding activity (B).

Individual variation in yields of STX binding activity (pmol/g tissue) was observed in several individuals (see Fig. 3.5). This evidence suggests that the component responsible for the STX binding activity may not be expressed consistently between individuals.

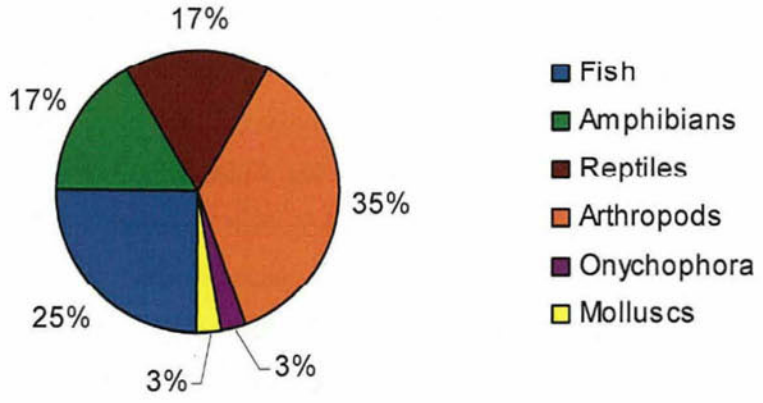

Figure 3.12 Current diversity of saxitoxin specific hydrophilic saxitoxin binding proteins now extends to phylum Onychophora and Mollusca.

Animals that were deemed negative in this and other studies could in fact have the potential to obtain such activity during different life stages and at different ages which will be examined in Chapter 5 for *Bufo marinus*. An environmental or biological trigger may also be required to initiate or down-regulate expression of the proteins. An obvious example of this requirement in other systems would be the heat shock proteins in corals which are up-regulated following exposure to increased water temperatures (Hayes and King, 1995; Tom et al., 1999). The requirement for an environmental or developmental trigger, may explain the lack of activity observed in animals closely related to species deemed positive in this and previous surveys. Animals may for instance, need to be exposed to STX or another unknown ligand in order to up-regulate the proteins which cannot be assured in any of the animals collected and tested in this particular study. Isolation of the STXBP genomic DNA of each positive animal would greatly assist in future screening efforts by enabling the development of specific molecular probes which could be used to amplify, isolate and sequence new STXBPs in the presence or absence of activity.

3.4.2 Structure-activity relationships between PSTs and novel STXBPs

The affinity of six novel and two previously identified STXBPs for 10 different PST derivatives was investigated during this study. PST affinity data collected for *B. marinus* plasma indicated that stoichiometric binding was optimal with minimal R1 and R3 substitution with dcSTX, STX, dcGTX 2,3 and NeoSTX exhibiting highest affinity for the STX binding component from this species. In most cases the addition of OSO_3^- at R2 or R3 reduced STXBP affinity, particularly in extracts from fish *B. rerio* and *G. hamiltoni* which could be due to steric or charge effects of the molecule and slight modifications of the binding site in these species. The addition of hydroxybenzoate group at R4 greatly reduced affinity in extracts from seven species, presumably due to steric hindrance at either 2 or 8-amino function (depending on 3D orientation of the R4 moiety), but this could only be confirmed following ligand receptor modelling or crystallography. In *L. elliptica* STX derivatives with substitutions at R2 and R3 demonstrated no competition with [^{3}H] STX in protein extracts whatsoever which suggests that the native 12, 12-diol form of the 4H, 12H pyrrolo group is critical to binding to the STXBP from this species. In contrast, toxins with R1 and R4 substitution made little difference to binding selectivity in extracts from this species suggesting that the toxins may bind in a different orientation or to a more open binding site than observed in other species.

In addition to gaining insight into the selectivity of different STXBPs for PSTs, these experiments validated the use of the MCE receptor assay developed in Chapter 2, for the detection and characterisation of the wider range of PST analogues. Appropriate configuration of the MCE binding assay for PST detection would require a sensitive STXBP substrate such as *B. marinus* plasma which is easily sourced from most countries and has a good sensitivity to all PSTs tested. The only drawback would be in the detection of C-toxins which was not examined during this study due to a lack of available standards, however *B.marinus* plasma is reported to have an IC_{50} of 7540 ± 620 nM for C1 (Llewellyn et al., 1997) but this value cannot be easily compared to the data accumulated during this study due to the change in assay format, differences in protein and [^{3}H] STX concentrations and altered experimental conditions used. Further examination of the effects of shellfish matrices should be examined prior to widespread use of

this method to ensure reproducibility and to determine detection limits in the presence of likely contaminants and solvents.

3.4.3 Classification of STXBP activity

STX binding activity was lost following tryptic digestion and sample boiling suggesting that all STX binding identified in extracts during the course of this study were proteinaceous. Subsequent estimation of protein size revealed three distinct size ranges of STX binding activity with the majority of species extracts identified between 50-100 kDa in size. The two exceptions included 1) *L. elliptica* with activity isolated between 20-50 kDa and 2) *Torquiguener pleurogramma* where activity was isolated between 100-200 kDa. Interestingly, bullfrog SXPN has been reported to be approximately 91 kDa in size (Lenarcic et al., 2000) which is clearly within the range of extracts from six species examined during this study. Other bi-lobed members of the transferrin family including serum transferrin, melanotransferrin, ovotransferrin and lactoferrin, are also within this size range (Lambert, 2004). Based on this information it is likely that the novel STXBPs identified 50 – 100 kDa in this study will also contain two lobes if in fact they are transferrin aberrants. Interestingly, a single lobe transferrin homolog has been identified in the ascidian, *Halocynthia roretzi* with the C-lobe observed in most transferrins missing and an estimated molecular weight of 52 kDa (Abe et al., 2001; Martin et al., 1984). The size range observed for *L. elliptica* is consistent with this single lobed transferrin variant, however based on the unique binding characteristics and affinities for some STX derivatives demonstrated by extracts from this species, it is inconclusive whether this STXBP is related to SXPN at all. Likewise the larger size observed in *T. pleurogramma* is consistent with a three-lobed transferrin sequences identified in marine algae, *Dunaliella salina* reported to be 150 kDa in size (Schwarz et al., 2003) and pacifastin (155 kDa) isolated from the crayfish, *P. leniusculus* (Liang et al., 1997). However, the identification of additional TTX binding activity of protein extracts of *T. pleurogramma* suggest that it is more likely that STXBPs from this species are related to the puffer-fish PSTBPs which were reported to be 106 kDa by SDS-PAGE (Yotsu-Yamashita et al., 2001).

In order to definitively classify STXBPs into their respective functional class i.e. VGSC, SXPN or PSTBP; each extract needs to be fully characterised, purified, sequenced and functionally expressed to confirm identity and relationships to these or other protein groups. In Chapter 5, the purification, characterisation and partial sequencing of a STXBP from *B. marinus* is described.

3.4.4 Co-occurrence of STXBPs and PSTs

All species screened for STXBPs were also extracted using the AOAC method to determine the presence of PSTs or TTX in the organisms by sodium channel and centipede SXPN radio-receptor assay. Any significant inhibition observed in one or both assays were confirmed by LC-FD. Of the 203 species tested ($n \geq 5$ for each species), extracts from only nine species inhibited the [^{3}H] STX binding to sodium channels, while seven were deemed positive for PST-like activity in the GF/B centipede SXPN assay (Table 3.7). LC-FD analysis of these extracts determined that PSTs were in fact present in four crab species (*Atergatis floridus, L. pictor, Liomera tristis* and *Thalamita crenata.*), two molluscs (*Tectus pyramis, Turbo argyrostomas*) and the toad fish *T. pleurogramma.* Low concentrations of TTX were confirmed by LC-FD in *L. pictor* (2-8 µg/100 g tissue), *Liomera tristis* (3-12 µg/100 g tissue) *and T. pleurogramma* (8-11 µg/100 g tissue). It cannot be discounted that toxins not examined in this study (e.g. C-toxin derivatives, diarrhetic shellfish toxins, ciguatera etc.) may have been present in any or all extracts tested; however, the hot acid extraction method should efficiently convert most C-toxins to gonyautoxins that would be detected by LC-FD. In addition, the relationship of non-PSTs with STXBPs has never been identified and therefore well beyond the scope of this study.

LC-FD quantitation of PSTs revealed STXeq concentrations above the public safe limit for the consumption of shellfish of 80 µg/ 100 g tissue (Van Egmond et al., 1992) in one *A. floridus* individual. The lack of evidence of PSTs in terrestrial extracts was not surprising, given that the known sources of PSTs include marine dinoflagellates and freshwater cyanobacteria, however controversial evidence of PST production in bacterial isolates from marine sources (Kodama et al., 1990) and in the anaerobic bacteria isolated from cattle rumen and grass (Sevcik et al., 2003) could not rule out this possibility. It should also be noted that there was no

evidence of active or recent algal blooms in the vicinity of any of the collection sites in this study so the level exposure of collected species to these toxins can not be determined. TTX on the other hand has been well documented in a variety of species from marine (Chen et al., 2002), terrestrial (Kim et al., 1975) and freshwater sources (Shin-Jung et al., 2002) so we had expected to observe a greater diversity of species containing this toxin. Of the toxic species, only *L. pictor* also exhibited STXBP activity. *L. pictor* is an acknowledged PST (Llewellyn and Endean, 1989; Tsai et al., 1995) and TTX vector (Teh and Gardiner, 1974; Tsai et al., 1995) and it has been previously demonstrated that this Xanthid crab contains a STXBP (Llewellyn, 1997). The coexistence of TTX and STX binding and LC-FD confirmation of TTX in the plasma of *T. pleurogramma,* suggests that the STXBP present in this species may be related to the PSTBPs isolated from other puffer-fish species (Yotsu-Yamashita et al., 2002; Yotsu-Yamashita et al., 2001). Future purification and sequencing of this protein should reveal the true relationship of STX binding activity observed in this species.

3.5 Conclusions

With the aid of the optimised MCE receptor assay, 203 species consisting of approximately 1000 individual extracts were screened for the presence of STXBPs. Of these, eight new STXBPs were identified extending the known phylogenetic diversity of STXBPs to include species from Onychophora and Mollusca. Six of these species were characterised as STX specific hydrophilic receptors based on their ability to exclusively bind STX and may be related to bullfrog SXPN; however, this classification should be revisited following purification, sequencing and functional expression of the proteins from individual species. In addition, a STXBP likely to belong to the PSTBP group was identified the toad fish, *T. pleurogramma.* Thorough application of the MCE receptor assay throughout this Chapter consolidated its utility for the sensitive and reliable detection and characterisation of STXBPs. Competitive binding assays of STX derivatives validated deployment of this technique for the detection of PSTs from biological extracts as suggested in Chapter 2. No clear relationship between the presence of PSTs and the expression of STXBPs was established within the confines of this study although it could not be discounted due to the lack of

evidence of exposure of these animals to STX sources. Examination of STXBP-producing species known to be exposed to STXs such as in a PST "hotspot" or following a "saxitoxic" algal bloom may provide clearer evidence of co-occurrence of ligand and receptor in nature.

3.6 Acknowledgements

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CHAPTER 4

Occurrence of paralytic shellfish toxins and saxitoxin binding proteins in a tropical saxitoxin "Hot Spot"

4.1 Introduction

In Australia, paralytic shellfish toxin (PST) production has been identified within a narrow range of micro algae including the cyanobacterium *Anabaena circinalis* and marine dinoflagellates *Alexandrium catenella, Alexandrium minutum, Alexandrium tamarensis* and *Gymnodinium catenatum* (Negri et al., 2003a). However, these studies have focussed on species originating from temperate regions of Australia such as Tasmania, South Australia and New South Wales, and very few species were collected or cultured from the more remote northern Australian coastline (Negri et al., 2003a). It is quite possible that additional tropical dinoflagellates such as *Pyrodinium bahamense* var *compressum,* which has been associated with paralytic shellfish poisoning (PSP) outbreaks in neighbouring countries of South East Asia (Azanza and Taylor, 2001; Bricelj and Shumway, 1998; Hallegraeff, 1993) may also inhabit Australia's northern waters.

PSTs can accumulate in filter feeders such as bivalves, which consume toxic dinoflagellates or cyanobacteria and the toxins can then be transferred to higher animals via the food web (see Fig 4.1). This accumulation can result in a significant public health threat and in turn have necessitated the development of regulations worldwide requiring testing of filter-feeding molluscs to ensure that contaminated produce is not consumed by the public (van Egmond et al., 1992). Currently acknowledged vectors of PST in the marine environment include mollusc (Morono et al., 2001; Sullivan and Iwaoka, 1983); crustacean (Negri and Llewellyn, 1998; Tsai et al., 1997); echinoderm (Ito et al., 2003; Lin et al., 1998) and fish species (Nakashima et al., 2004).

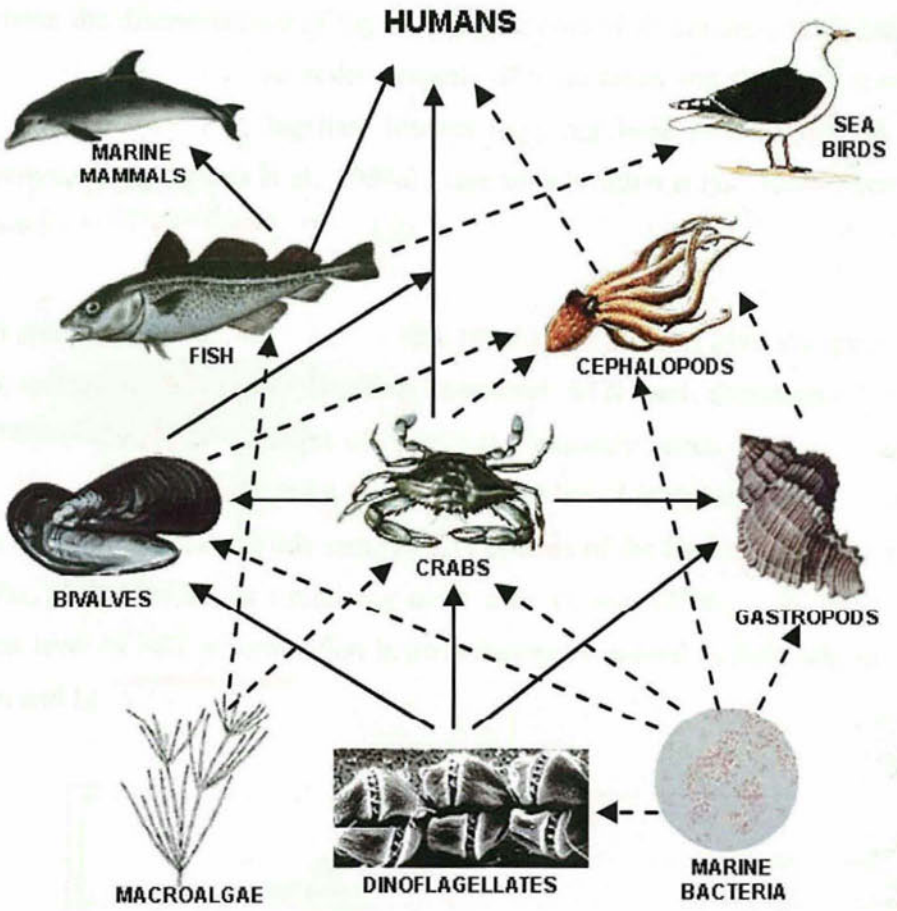

Figure 4.1 Representative marine food web. Filter feeding shellfish consume toxic phytoplankton and accumulate paralytic shellfish toxins which are then transferred to higher trophic levels. Deaths resulting from paralytic shellfish poisoning have been recorded in humans, marine mammals and fish. Known pathways are indicated by solid lines while dashed lines indicate proposed or assumed pathways.

At higher levels of the food web, saxitoxin (STX) intoxications can result in significant impacts, with fish (Robineau et al., 1991; White, 1984), dolphin (Hokama et al., 1990), seal (Reyero et al., 1999) and indeed human (Llewellyn et al., 2002) deaths attributed to consumption of PST vectors. Although widely accepted, there are studies that contradict the food web bioaccumulation dogma, for example: shellfish can become highly toxic during blooms of *A. catenella*, despite a lack of toxicity in the algal cells (Sakamoto et al., 1992).

Toxicity in the scallop *Patinopecten yessoensis* only increased significantly following the disappearance of high density blooms of *A. tamarensis* (Sakamoto et al., 1992). There are also several reports of toxic crabs and shellfish in warm waters where toxic dinoflagellate blooms have not been reported (Negri and Llewellyn, 1998; Oshima et al., 1984a). One such location is Port Hedland on the north-west coast of Australia (Fig. 4.2).

Negri and Llewellyn (1998) reported that 19 of 20 individuals from six species of crabs examined from Port Hedland contained STX and decarbamoyl STX (dcSTX) including four species of crabs not previously acknowledged as toxic, making the port one of the most 'saxitoxic" sites tested in the northern Australia region at that time. During this same study a species of the Xanthid crab *Atergatis floridus*, was recorded as containing more than 16 mg STXeq/100g tissue, the highest level of PST accumulation in a crustacean on record in Australia to date (Negri and Llewellyn, 1998).

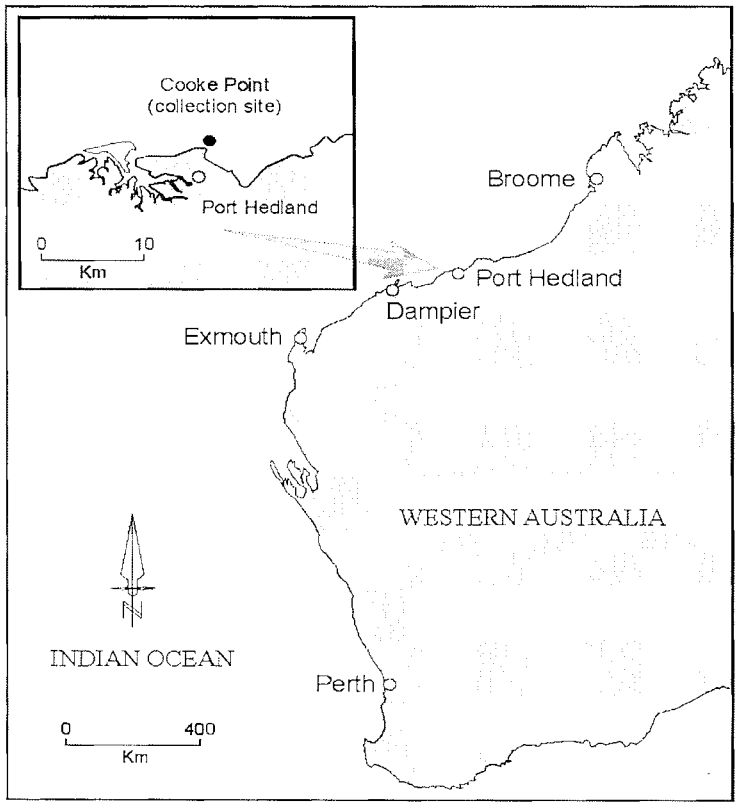

Figure 4.2 Map of Western Australian coastline with inset highlighting study site at Cooke Point, Port Hedland.

The source of PSTs in Port Hedland, is yet to be determined but strong circumstantial evidence from other regions of Australia, namely Tasmania, indicate that exotic dinoflagellate species such as *Gymnodinium catenatum* may have been introduced into southern waterways via ballast water discharge from international vessels (Hallegraeff and Bolch, 1991; Hallegraeff and Jeffrey, 1984). Port Hedland is the 3rd largest tonnage port in Australia (PHPA, 2003) so it is conceivable that a tropical dinoflagellate species such as *Pyrodinium bahamense* could have been introduced into the surrounding waters or sediments of Port Hedland via ballast water, however there have been no records or evidence of blooms of these or any other dinoflagellates in the area (Reid *Pers. Comm*). In one survey, several species of *Gymnodinium* were found to be dominant in the phytoplankton biomass from the water column along the North West Shelf of Australia, but it was not determined whether these species contained toxic strains (Hallegraeff and Jeffrey, 1984). Likewise, in 1998, a survey for introduced pests failed to identify toxic dinoflagellate species in the port or adjacent coast although a *Gymnodinium* species of unknown toxicity was identified at some sites (Hewitt et al., 1999). Hallegraeff, (1998) identified *Alexandrium catenella* in ballast water from Port Hedland but again there was no evidence of PST producing plankton dinoflagellates during the comprehensive survey of the area (Hallegraeff, 1998) or at any Queensland tropical ports (Hallegraeff, *Pers. Comm*). Alternatively, it is possible that the source of PSTs in the benthos at Port Hedland may be attributed to macro algae such as *Jania* sp, which has been linked to PST accumulation in Japan (Kotaki et al., 1983; Oshima et al., 1984a), and may explain the accumulation of PSTs in Xanthid crabs from Port Hedland. However, a macro algal source of PSTs would not clarify the source of accumulation in filter feeding organisms along the tropical coastline.

In the marine environment, several vectors of PSTs appear to have developed a variety of strategies to protect themselves from the effects of these potent neurotoxins. In some crustaceans such as *Atergatis floridus*, which has been shown to accumulate STX and tetrodotoxin (TTX) in its tissues (Arakawa et al., 1995; Raj et al., 1983; Tsai et al., 1995), evidence of nerve resistance to the effects of PSTs has been reported (Daigo et al., 1988) suggesting that affinity for PSTs in the sodium channels of these species is greatly reduced. Likewise, nerve

resistance to STX has also been reported in molluscan species also known to harbour PSTs (Kvitek and Beitler, 1991).

Despite evidence of STX resistant nerves in these species, not all marine animals capable of STX accumulation share this biological advantage but appear to have acquired other mechanisms of survival. Several species of Xanthid crabs including *Lophozozymus pictor, Liomera tristis, Chlorodiella nigra* and *Actaeodes tomentosus*, collected from the Great Barrier Reef in Queensland, Australia were reported to contain STX specific binding activity in their hemolymph (Llewellyn, 1997; Llewellyn et al., 1997) which may act as a STX resistance mechanism in these animals. While the biological role of STXBPs is yet to be identified, it is possible that the presence of STXBPs in PST vector species may act either as a first line of defence against intoxication by PSTs or in actual transport and or excretion of the toxins from the organism as postulated by Llewellyn (1997). However, as with nerve resistance, this STX binding hemolymph protein has not been identified in all PST vectors and does not appear to be present in species such as *Atergatis floridus* which exhibit STX resistant nerves (Llewellyn, 1997).

Evidence of STX specific binding proteins in other benthic marine animals such as the mollusc, *Laternula elliptica* has been identified (see Chapter 3), however individual variation in protein yields and specific activities between individuals was observed, suggesting that an environmental or biological trigger may be required to promote expression of the protein (Robertson, Chapter 3). A reasonable hypothesis would be that STX exposure is the trigger and if this is the case, animals equipped with STX binding machinery would be expected to reside in geographical sites of high STX prevalence.

4.1.1 Aims of this study

Port Hedland is clearly a tropical PST 'hot spot', although the sources of STX derivatives remain a mystery. An area of such high STX prevalence provided a setting for the examination of STXBP occurrence and to test the hypothesis that these unique soluble receptors play a role in the protection or facilitation of PST bioaccumulation in the benthic food web. The likelihood of PST exposure in benthic animals is very high, so if STXBP activity is invoked by a trigger of this

kind we would expect to find a variety of animals that exhibit STX binding activity. In addition, identification of new sources or vectors of PSTs may help to explain the diversity of STXBPs, which may in turn assist in the understanding of their possible biological function.

The specific aims of this chapter were to:

- Collect a variety of species from representative levels of the benthic food chain including macro-algae, gastropods, bivalves, crustaceans and octopi.
- Investigate the occurrence of STX-like activity in the extracts of animals of a benthic food chain by radio-receptor assay.
- Examine tissue distribution of PSTs in any species not previously identified as vectors of PSTs.
- Identify toxin profiles of PSTs by liquid chromatography (LC-FD).
- Confirm structural identities of toxins in new vector species by liquid chromatography- mass spectrometry (LC-MS).
- Assess the occurrence of saxitoxin binding proteins in organisms by protein binding [^{3}H] STX binding radio-receptor assay.
- Characterise STX binding and determine saturation binding parameters in all species identified activity.
- Examine the co-occurrence of PSTs and STX binding proteins in individual benthic species.

4.2 Materials and Methods

4.2.1 Collection from Cooke Point, Port Hedland, Australia

A variety of benthic invertebrates representing a simple food web were hand collected from a reef flat at Cooke Point, Port Hedland in the Pilbara region of Northern Western Australia (20°19'S, 118°34'E; Fig. 4.2). Two different collections were examined from the site at Cooke Point during this study including a variety of macroscopic algae, gastropods, bivalves, crabs and one species of octopi, *Octopus* (*Abdopus*) sp. 5 (Norman, 2000); Fig. 4.3) collected by Drs. Andrew Negri and Lyndon Llewellyn on 22nd October, 1997 and stored at -20°C until extraction and analysis during this study initiated in 2001 (collection A).

An additional collection of targeted species of *Octopus (Abdopus)* sp. 5 (Norman, 2000) and crustaceans including *Atergatis floridus, Pilumnus pulcher, Thalamita stimpsoni* and *Lophozozymus octodentatus* (Fig. 4.4) were collected in July 2002 with the assistance of Karin and Graeme Wignell from Big Blue Dive, Port Hedland (collection B). Details of collection A and B are provided in Table 4.1.

Prior to storage and transport, all animals were humanely sacrificed according to the JCU ethical guidelines for the use and handling of animals (Permit No.A745-02). All shelled molluscs and crustaceans were anaesthetised by cooling to 4°C, then stored frozen at -20°C. Octopi were euthanased by decapitation through the brain (i.e. severed across the mid section of the head between the buccal mass and mantle) with heavy duty shears to ensure immediate immobilisation and rapid death. Octopi were then stored at -20°C. The octopi had an average body size to 3-6 cm and tentacle span ranging between 15 and 30 cm. Frozen samples were shipped on ice to laboratories at AIMS in Townsville, Queensland Australia.

Table 4.1 Species collection summary from Cooke Point, Port Hedland, Western Australia.

Species	Collection A Oct 1997	Collection B Jul 2002
Algae		
*Stoechospermum sp.**	1	-
*Sargassum sp.**	1	-
Jania sp. 2 *	1	-
Jania sp. 3 *	1	-
Molluscs		
Trochus hanleyanus	4	-
Tectus pyramis	4	-
Tridacna squamosa	4	-
Pinctada albina sugilata	4	-
Malleus (Malvufundus) regula	4	-
Saccostrea glomerata	4	-
Octopus (Abdopus) sp. 5	4	12
Crabs		
Lophozozymus octodentatus	4	8
Atergatis floridus	4	8
Thalamita stimpsoni	4	8
Pilumnus pulcher	4	6

- denotes that no animals of this species were collected at the time specified

* pooled samples of at least 6 individuals

4.2.2 Paralytic shellfish toxin extraction: Collection A

All samples from the collection A were extracted for PSTs by a cold extraction method as previously described (Llewellyn et al., 2002). Prior to extraction molluscs were removed from their shells, while macro-algae and crabs were blended whole. Individual samples were then divided into two equal sub-samples with one retained for later protein extraction. 3 legs from each of the 4 individuals of *Octopus (Abdopus)* sp. 5 from collection A were pooled for extraction. All samples were extracted with 80% ethanol (pH 2, HCl), using a ratio of 5 mL/ g tissue and this mixture was homogenised firstly in a Waring blender (2 × 20 sec. bursts at maximum setting) followed by finer homogenisation on ice using a PRO250 tissue disruptor fitted with a 10 mm toothed head (2 x 15 second burst at 8,000 rpm, followed by a single 15 second burst at 24,000 rpm).

Each extract was sonicated three times for 10 min in a Soniclean 500T bath sonicator (Transtek Systems, Selby Biolab), resting on ice for 10 min between sonic treatments, then clarified by centrifugation at 10,000 x g for 20 min at 4°C, in a Hermle Z323K refrigerated centrifuge. The process of homogenisation, sonication and centrifugation was repeated two additional times with fresh extraction solvent. For each extract, the three supernatants generated were pooled and hydrophobic components separated by partitioning in a separating funnel with dichloromethane. Hydrophilic fractions (that potentially contain PSTs) were lyophilised and reconstituted in 0.05 M acetic acid. This mixture was then applied to a 500 mg Extract-Clean C-18 sep-pak (Alltech Associates, NSW, Australia) with 0.2 μm filter attached (Minisart, Sartorius, Gottingen, Germany). The sep-pak was pre-equilibrated with 10 mL methanol, followed by 20 mL of 0.05 M acetic acid prior to the application of the sample. Eluate was collected along with a 5 mL wash with 0.05 M acetic acid, then dried *in vacuo* and redissolved into 1 mL 0.05 M acetic acid. This sample was then passed through a 10,000 MWCO centrifugal ultrafilter (Ultrafree-MC, Millipore, Sydney, Australia) to yield samples for bioassays and LC-FD.

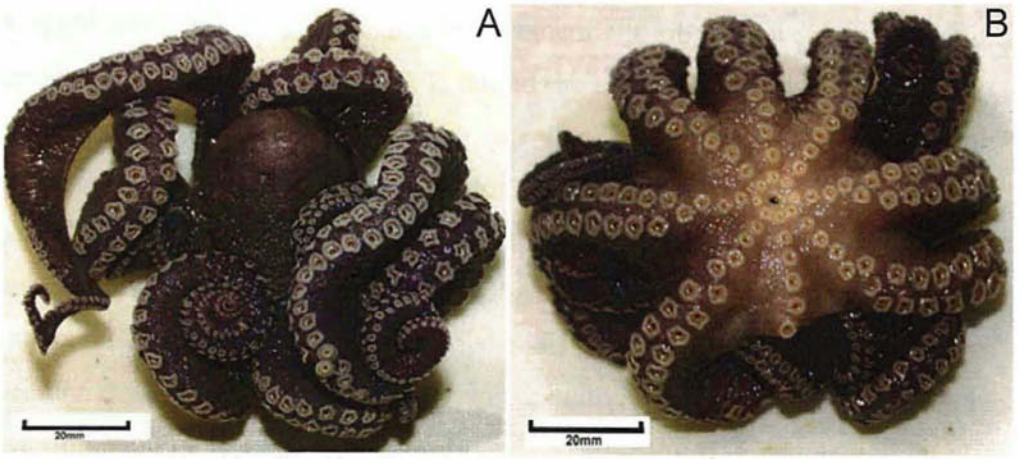

Figure 4.3 Dorsal (A) and ventral (B) view of an octopus specimen collected from Cooke Point, Port Hedland, Western Australia identified as *Octopus (Abdopus)* sp. 5 (Norman, 2000).

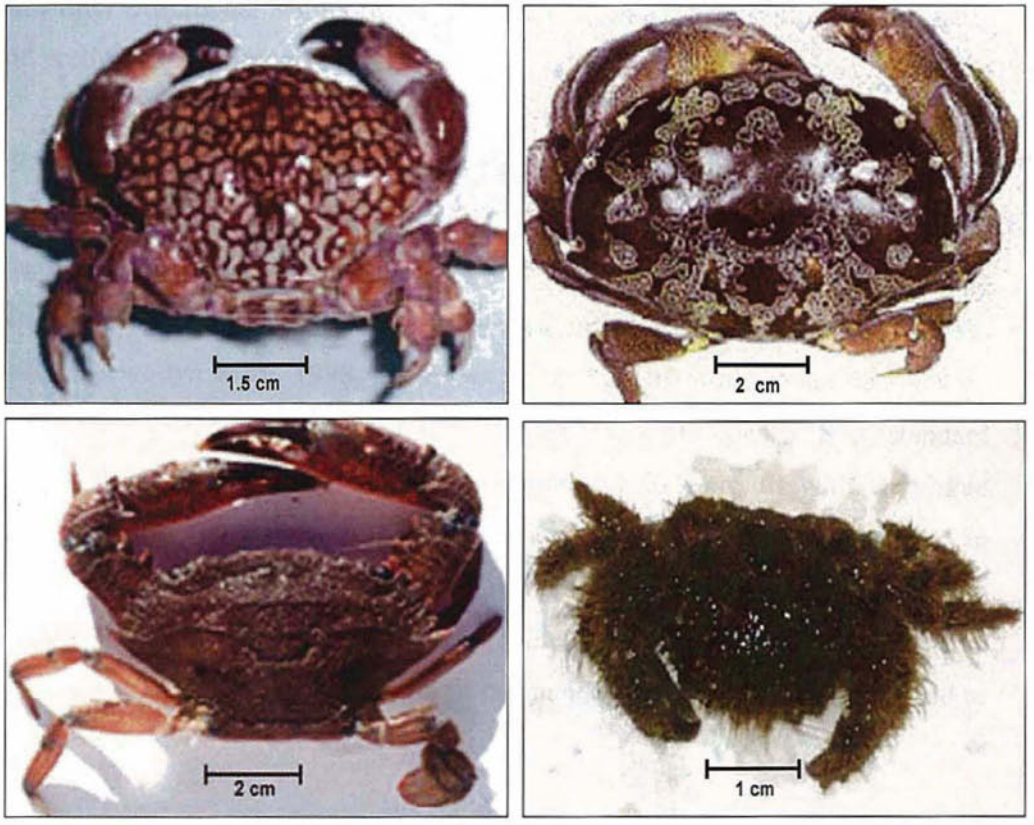

Figure 4.4 Photographs of crab species targeted in 2002 benthic collection from Cooke Point, Port Hedland. Species included *Lophozozymus octodentatus* (A), *Atergatis floridus* (B), *Thalamita stimpsoni* (C) and *Pilumnus pulcher*.

4.2.3 Paralytic shellfish toxin extraction: Collection B

A slight modification in the extraction protocol described above was performed for all individuals from collection B due to the timely discovery of hydrophobic saxitoxin derivatives in *Gymnodinium catenatum* (Negri et al., 2003b). Animals from collection B were extracted for PSTs omitting the dichloromethane partitioning and C18 sep-pak filtration steps. Prior to extraction, individual samples were divided into two equal sub-samples with one retained for protein extraction. All crabs and six octopi specimens were homogenised whole with an additional six octopi dissected to isolate individual organs and tissues for determination of tissue distribution of any PSTs identified. Homogenisation was performed three times as described for collection A, with the three supernatants generated pooled and filtered through 0.2 µM nylon filters (Millipore, North Ryde, NSW, AU) and lyophilised. Dried extracts were reconstituted in 0.05 M acetic acid (1.0 mL) and extracts passed through a 10,000 MWCO centrifugal ultra-filter (Microcon, Millipore). Ultra filtrates then used for radio-receptor assay and preliminary chemical analysis.

4.2.4 Sodium channel and saxiphilin radio-receptor assay

Quantitation of STX concentration equivalents (STXeq) with [^{3}H] STX radio-receptor assays using saxiphilin (SXPN) and rat brain synaptosomes, was performed as described elsewhere (Llewellyn and Doyle, 2001; Llewellyn et al., 2001a; Llewellyn et al., 1998; Llewellyn et al., 2001b). Calibration competition curves were generated between 1 nM [^{3}H] STX and dilutions of STX standard ranging from 0.06 nM-1.67 µM. Competition curves were fit with GraphPad Prism 3.0 (GraphPad Software Inc, San Diego, CA). Extracts were assayed in triplicate for inhibition of STX binding in both the sodium channel (NaCh) and centipede SXPN assays. Active extracts were then titrated to obtain a fraction bound value within the linear portion of the standard curve, and then converted to STXeq values with the equation:

$$\left(\frac{100-F}{F}\right)^{1/n} \text{ x } IC_{50}$$

where F was % [^{3}H] STX bound in the presence of extract compared to controls; n = Hill slope of the non-linear regression curve, and IC_{50} was the concentration of

STX which caused 50% inhibition in the standard curves. Calculations were then adjusted to account for dilution factors from extraction and assay.

4.2.5 High performance liquid chromatography - fluorescence detection

All sample extracts which competed with [^{3}H] STX in the radio-receptor assays were analysed by high performance liquid chromatography – fluorescence detection (LC-FD) for STX, decarbamoyl STX, neoSTX, 11-hydroxysulphate STX's (GTX 1-6), N-sulphocarbamoyl-11-hydroxy-sulphate STX's (C-toxins 1-4), hydroxybenzoate STX's (GC 1-3); and TTX. LC-FD was performed using a Waters 600 HPLC (Milford, MA, USA) coupled to a Linear LC-305 fluorescent detector (Activon), Waters 717plus auto-sampler and a PCX 5100 Post-Column reactor (Pickering, Mountain View, CA, USA). PST's were eluted as previously described (Negri and Llewellyn, 1998; Oshima et al., 1984b) while GC toxin analogues were analysed as recently reported (Negri et al., 2003b). Fluorescent derivatives of all PST's were monitored at $\lambda_{excitation}$= 330 nm and $\lambda_{emission}$= 390 nm. This technique can be compromised by impostor peaks possessing fluorescence properties akin to the PST's (Baker et al., 2003). To eliminate this possibility, all suspect peaks were interrogated using spiking experiments, measurement of emission maxima and detection in the absence of post-column oxidation (Onodera et al., 1997). TTX derivatives were analysed using a 5 μm 250 mm × 2.1 mm Alltech Alltima C-18 column, eluted isocratically with 10 mM heptane sulfonic acid, then oxidised post-column in 4 M NaOH and monitored at $\lambda_{excitation}$= 415 nm and $\lambda_{emission}$= 485 nm (Yotsu et al., 1989).

4.2.6 Paralytic shellfish toxin purification

An extract of *Jania* sp.3 and *S. glomerata* from collection A, in addition to highly toxic specimens of *L. octodentatus* and *A. floridus* from collection B, deemed positive for PSTs by radio-receptor assay and LC-FD, were purified by chromatography to resolve PST related compounds by LC-FD. The toxins were eluted on a 1 cm × 25 cm Bio-Gel P-2 column (Bio-Rad, NSW) with 0.05 M acetic acid, then on a BioRex column of similar dimensions (1 cm × 28 cm; BioRad) with a 0-2 M gradient of acetic acid. Active fractions were determined by

centipede SXPN assay and pooled prior to evaporation in vacuo at room temperature. Samples were then re-analysed by LC-FD.

4.2.7 High performance liquid chromatography-mass spectrometry (LC-MS)

Confirmatory LC-MS analysis was performed on extracts of individuals from *Octopus (Abdopus)* sp. 5. Identification of suspect toxin peaks was undertaken by mass spectrometric analysis of separated toxins using LC-MS performed in single ion monitoring (SIM) mode. A Shimadzu LC-10ADVP liquid chromatography system and Gilson 233XL sampling injector (Gilson, Inc. Middleton, WI, USA) were coupled to a PE-SCIEX API 2000 triple quadrupole mass spectrometer (PE-SCIEX, Thornhill, Ontario, CA). LC separation was achieved on a TSK-Gel Amide-80 column (5 µm, 250 mm x 4.6 mm i.d. TosoHass) maintained at 40°C with an isocratic solution of 2mM ammonium formate, 3.6 mM formic acid in 70% acetonitrile:water at 0.2 mL min^{-1} and flow was introduced into the turbo ion spray interface with a 9:1 (waste:MS) split (Quilliam, 2001). Retention times of STX and TTX standards were compared to sample extracts.

Further LC-MS/MS experiments were performed using an Agilent 1100 Series LC coupled to an Esquire 3000+ quadrupole ion-trap mass spectrometer (Bruker Daltonics, Billerica MA, USA) fitted with an electrospray ionisation interface (HV capillary +4 kV, skimmer voltage 40 V). STX analytical standards and octopi extracts (in 5% acetic acid) were analysed individually using a TSK-Gel Amide-80 column (5 µm, 250 mm x 4.6 mm i.d. TosoHass) maintained at 40°C under the same mobile phase and experimental conditions as indicated above. Precursor ions at m/z = 300 Da were isolated (isolation width 6 m/z units), automatically fragmented and daughter ions scanned between 100 and 400 Da. Daughter ion mass spectra of sample and authentic STX standard were then compared and exploited for parent ion mass loss interpretation.

4.2.8 Protein extraction

All molluscan and crustacean specimens collected during the course of this study were divided into two sub-samples prior to extraction with one half of the sample extracted by PST protocol described above and the other half retained for protein extraction. These samples included all molluscs and crustaceans from collection A

and *Octopus (Abdopus)* sp. 5 ((Norman, 2000); Fig 4.4), *A. floridus, P. pulcher, T. stimpsoni* and *L. octodentatus* (Fig. 4.5) from collection B. The carapace was removed from the animals prior to rapid freezing in $N_{2\ (l)}$, then tissues were ground to a powder by mortar and pestle. Powdered samples were then reconstituted in 20 mM HEPES-NaOH (pH 7.4) containing a cocktail of protease inhibitors (cØmplete, Roche) at a ratio of 2 mL buffer /g and vortexed on ice. Extracts were then filtered through 0.2 µM cellulose acetate filters (Millipore), centrifuged for 20 min. at 15 000 × g, 4°C (Hermle Z323K refrigerated centrifuge) to remove membrane materials potentially containing sodium channels and stored at -80°C until assay.

4.2.9 MCE protein binding [^{3}H] STX binding assay

Saxitoxin binding activity of all protein extracts of Port Hedland molluscs and crustaceans were assessed by MCE Protein binding [^{3}H] STX binding assay developed in Chapter 2 and was performed as detailed in Chapter 3. Duplicate aliquots of standardised protein extracts (50 µg total protein) were incubated in the presence of 2 nM [^{3}H] STX, 50 mM NaCl, 1 mM EDTA and 20 mM HEPES-NaOH, pH 7.4 in a final volume of 100 µL at room temperature for 60 min. Millipore Multiscreen™ mixed cellulose ester filter plates were pre-wet with 20 mM-HEPES-NaOH, pH 7.4 for 2 min. then aspirated prior to the addition of pre-equilibrated samples. Samples were allowed to equilibrate to the filters for 5 min. and were then washed three times with 20 mM HEPES; 1 mM EDTA, pH 7.4 (4°C). Filters were punched into scintillation counting plates (Perkin Elmer) and 200 µL Optiphase Supermix scintillant added. Plates were then sealed and shaken for 30 min. followed by liquid scintillation counting in a Wallac MicroBeta Liquid scintillation counter (Perkin Elmer). Non-specific binding was monitored by incorporation of 5 µM unlabelled STX as previously described (Chapter 2, 3).

4.2.10 Characterisation of STX binding activity

Binding site titrations and saturation binding curves with [^{3}H] STX concentrations ranging from 0.1 to 25 nM were generated for all extracts deemed positive and protein quantitation was performed using the BioRad protein assay as detailed in Chapter 3. Specific activity of all extracts of any species identified as positive for STX binding activity was determined to gauge individual variation of STXBP

expression in that animal. Fe^{3+} and TTX inhibition of [^{3}H] STX binding examined displacement assays and competitive binding experiments in all active extracts as detailed in Chapter 2 and 3 of this thesis.

Pooled extracts of individuals of a single species exhibiting STX binding activity were pooled to form a single extract then protein concentration and activity was re-quantified. Several aliquots of pooled protein extracts were then boiled and additional aliquots digested with trypsin to determine whether saxitoxin activity was derived from a proteinaceous component of the extract, as described in Chapter 3. Finally, assay guided size estimation of all extracts positively identified with STX binding activity was performed by ultrafiltration with centrifugal molecular weight sieves of various sizes (200 kDa; 100 kDa; 50 kDa; 20 kDa; 10 kDa; Millipore) as previously reported (Chapter 3).

4.3 Results

4.3.1 Detection of PSTs by radio-receptor assays and LC-FLD

STX-like activity in molluscan and crustacean extracts from Cooke Point, Port Hedland (Fig 4.2) was initially determined using NaCh and SXPN [^{3}H] STX radio-receptor assays. Toxin concentration in each extract was calculated from calibration curves using Hill slopes of 1.15 and 1.2 and IC_{50} of 1.2 nM and 1.5 nM for the NaCh assay, and SXPN receptor assays, respectively (see Fig. 4.5). The presence and identification of PSTs was determined by LC-FD analysis whereby the retention of calibrated standards were compared to sample extracts. All suspect peaks were then interrogated further by spiking experiments, analysis in the absence of post-column reaction and emission scanning.

In some instances peaks were identified with dissimilar retention time to any of the standards tested (C-toxin 1-4; GTX 1-6; STX; dcSTX; neoSTX; dcGTX 2,3; GC1-3) but met the PST LC-FD criteria of $\lambda_{emission}$= 390 nm and disappearance of fluorescent peaks in the absence of post-column reaction. In these cases, identification of the peak was not possible by LC-FD and extract activity in radio-receptor assays determined level of toxicity and or likelihood of PSTs.

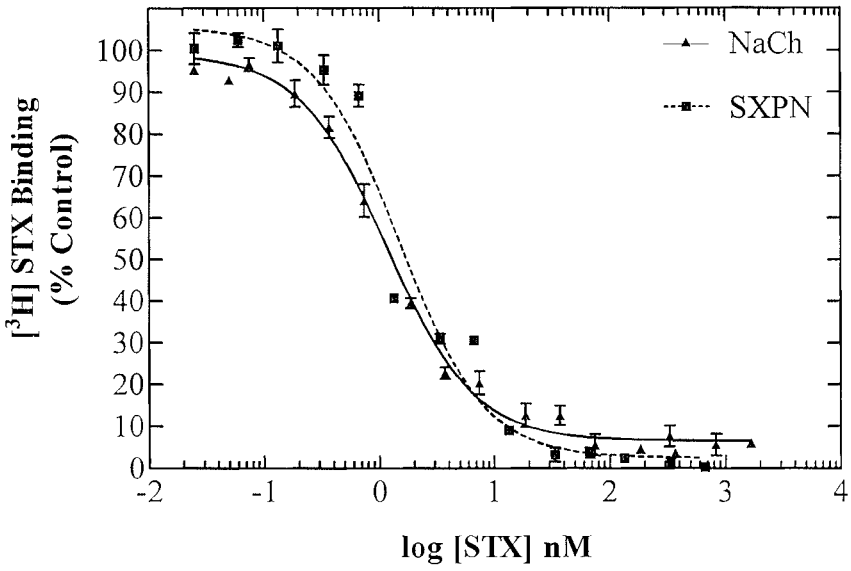

Figure 4.5 Assay calibration curves of competitive binding between STX and [^{3}H] STX, to rat brain sodium channel (▲ NaCh) and centipede saxiphilin (■ SXPN). Data points are the mean and standard error of triplicate measurements.

Total toxin concentrations were converted to STXeq and of the 84 extracts tested during the course of this study 82 were identified with PST activity by at least one method. 40 of the 84 animals tested, exceeded the regulatory limit for shellfish (80 µg STXeq/100g tissue) represented by 23 extracts from collection A (Table 4.2) and 17 extracts from field collection B (see Appendix 3).

Quantitation of STXeq by NaCh assay, centipede SXPN assay and LC-FD correlated well with linear regressions resulting from comparison between SXPN *vs.* NaCh; LC-FD *vs.* SXPN; and LC-FD *vs.* NaCh resulting in correlation coefficients (r^2) of 0.99; 0.98 and 0.98, within 95% confidence intervals respectively (Fig 4.6).

Evidence of PSTs in macro-algae

PST activity was identified in all four species of macro-algae tested however only the two *Jania spp.* and *Sargassum sp. 1* were confirmed by LC-FD. PST levels in *Jania Sp. 2* and *Jania Sp. 3* were estimated at 16 µg STXeq /100 g and 23 µg STXeq /100 g, respectively by LC-FD quantitation (see Table 4.2). Extracts of

Jania spp. were confirmed to contain GTX 1,4 and GTX 2,3 epimer pairs (Table 4.3). Further purification of *Jania sp. 3* confirmed these findings but did not reveal any additional PSTs (see Fig 4.7C). Trace amounts of STX (0.6 µg STXeq /100 g by LC-FD) was also identified in the extracts of the brown alga, *Sargassum sp. 1* (see Table 4.2; Table 4.3).

Evidence of PSTs in gastropod and bivalve molluscs

Two gastropod species from the family Trochidae were collected and extracted for PSTs during the course of this study as detailed in Table 4.1. The presence of PSTs was confirmed in all four individuals of *Trochus hanleyanus* and contained levels in the order of 1.8- 21 µg STXeq /100 g tissue as determined by LC-FD analysis (Table 4.2). Likewise, all 4 extracts of *Tectus pyramis* were deemed toxic and 3 exceeded the regulatory safe limit of 80 µg STXeq /100 g tissue with concentrations calculated at 18-154 µg STXeq /100 g tissue by LC-FD (Table 4.2).

Toxin profiles of these animals confirmed the presence of STX and dcSTX in most instances in addition to GTX 1, GTX2, GTX3 and GTX4 observed in some individuals (Table 4.3). 12 extracts from a total of 16 bivalves from four different species were identified with STX-like activity by radio-receptor assay and LC-FD with 10 animals within the regulatory limit for the safe consumption of shellfish (Table 4.2).

Saxitoxin was identified as the major contributing toxin in most toxic bivalves analysed by LC-FD and several of these also contained dcSTX (Table 4.3). An extract of *Saccostrea glomerata* (extract 23) was partially purified to establish a clear toxin profile by LC-FD and was clearly shown to contain dcSTX, STX, and the sulphated carbamate epimers GTX 2 and GTX3 (Fig 4.7E, F).

Table 4.2 Sodium channel (NaCh) and centipede saxiphilin (SXPN) radio-receptor assay and LC-FD quantification of toxins identified in extracts from algae and molluscs from Port Hedland collection A.

Species	ID	µg STX equivalents/ 100g tissue†		
		NaCh	SXPN	LC-FD
Algae				
Stoechospermum sp. *	1	2	1.1	-
Sargassum sp. 1 *	2	3.0	1.2	0.6
Jania sp. 2 *	3	18	5.9	16
Jania sp. 3 *	4¶	15	19	23
Molluscs				
Trochus hanleyanus	5	104#	54	18
	6	13	6.4	21
	7	33	2.5	1.8
	8	120#	63	16
Tectus pyramis	25	178#	98#	102#
	26	329#	176#	154#
	27	43	34	18
	28	180#	108#	121#
Tridacna squamosa	9	4.7	3.2	1.8
	10	8.9	6.0	7.5
	11	12	9.4	29
	12	-	-	-
Pinctada albina sugilata	13	74	140#	62
	14	-	1.2	-
	15	4.5	5.1	8.3
	16	13	12	30
Malleus (Malvufundus) regula	17	98#	59	36
	18	78	51	44
	19	23	66	-
	20	12	6.5	4.7
Saccostrea glomerata	21	35	42	28
	22	18	12	-
	23¶	101#	84#	67
	24	10	8.0	6.5

† STX equivalents calculated as described in "Methods' and equated to µg/100 g organism.
* Pooled samples of at least 6 individuals
# Toxicity > 80 µg STXeq/100 g tissue, exceeding public safe limit for the consumption of shellfish
¶ LC-FD spectra provided in Figure 4.9.
- Below detectable limits

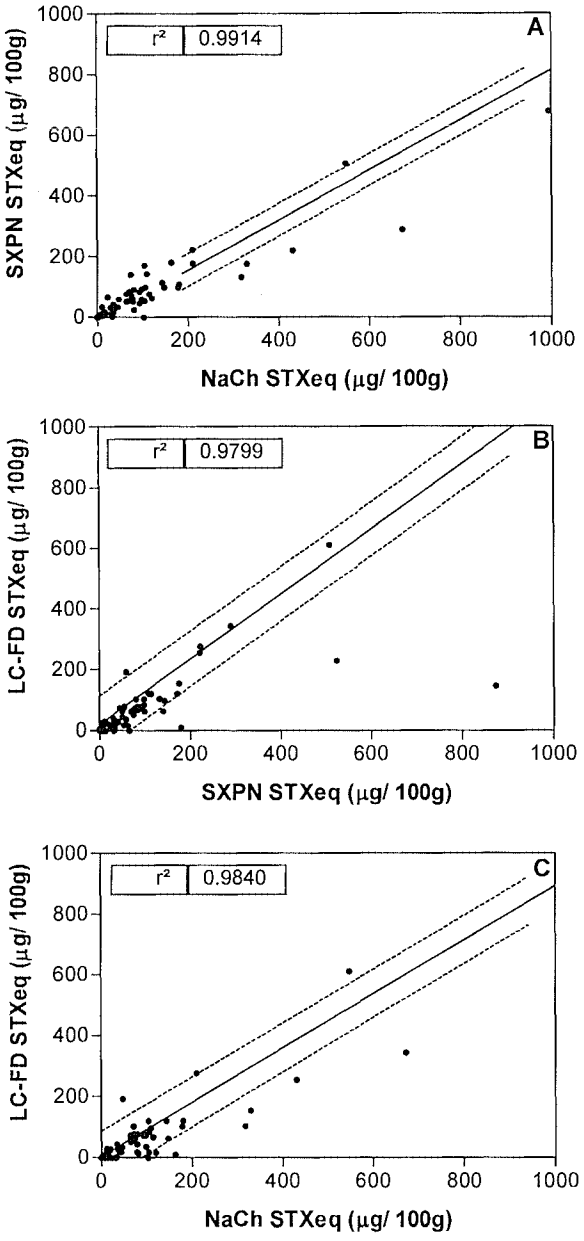

Figure 4.6 Correlation between sodium channel (NaCh) and centipede saxiphilin (SXPN) radio-receptor assays and LC-FD analysis (LC-FD) of the total toxin concentrations of extracts of algae, molluscs and crustaceans from PortHe dland. Data were calculated as described in "Methods" and are expressed as STX equivalents (STXeq) µg/ 100 g tissue. All extracts deemed positive for PSTs from collections A and B are included in the analysis however the range has been reduced for clarity of data points below 1000 µg STXeq/ 100 g. Solid line in each case is the linear regression with R^2 values located in the inset. Dashed lines represent the 95 % confidence intervals of the linear regression.

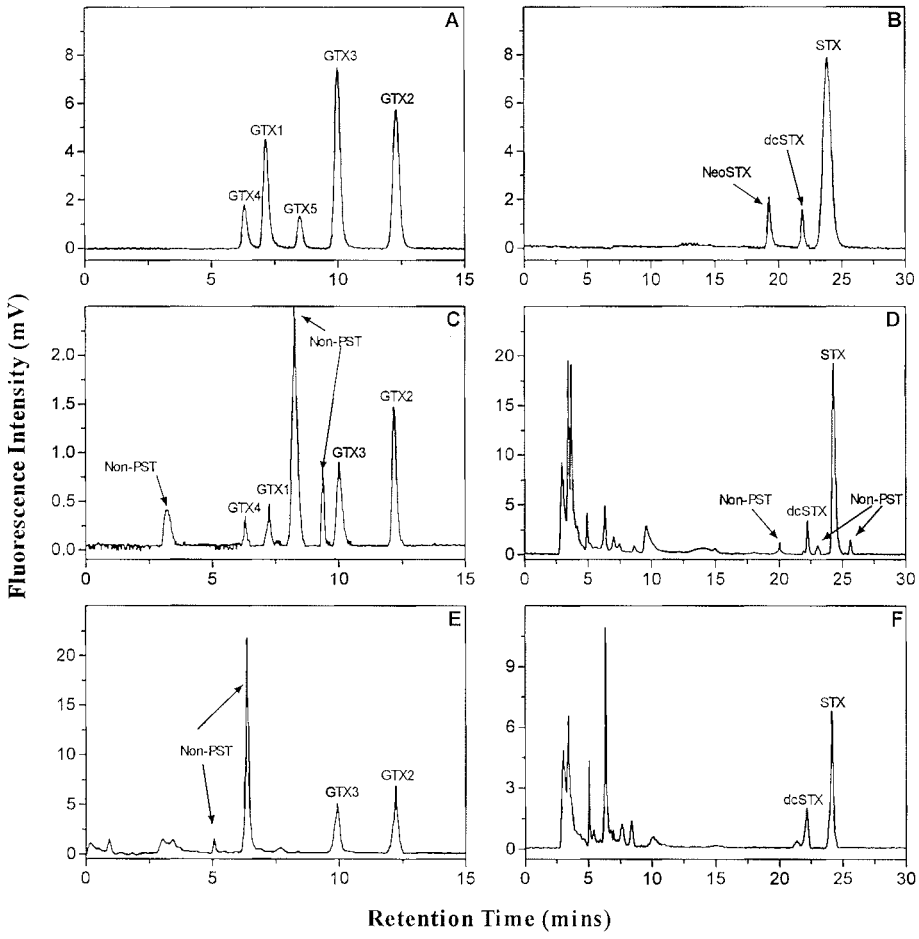

Figure 4.7 Several examples of LC-FD analyses of PSTs from organisms collected from Cooke Point, Port Hedland. Conditions suitable for the examination of gonyautoxins 1-5 were used to obtain trace A, C and E. Analyses targeting saxitoxins are shown in traces B, D and F. Trace A is a standard injection of a gonyautoxin mixture containing gonyautoxins 1-5 and trace B shows the retention of a mixture of calibrated standards consisting of Neo-saxitoxin, decarbamoyl saxitoxin and saxitoxin. A purified fraction from the algae*J ania* sp. 3 (extract 4) was confirmed to contain gonyautoxin derivatives 1,2,3 and 4 as shown in trace C. Trace D depicts PSTs identified by LC-FD for the Xanthid crab*L ophozozymus octodentatus* (extract 50). Trace E and F show the PST derivatives identified in the purified extracts of *Saccostrea glomerata* (extract 23). Peaks labelled non-PSTs were shown not to be PSTs based on peak emission scanning, spiking experiments and by their disappearance after eliminating post-column oxidation.

Evidence of PSTs in crustaceans

In general, extracts of crustaceans collected from Cooke Point in October 1997 (collection A) yielded higher toxin concentrations than animals collected in 2002 (Fig 4.8; Fig 4.9A). Specimens of *Atergatis floridus* recorded the highest levels of STX activity with levels up to 10,029 µg STXeq /100 g tissue equating to over 10 mg STX/100 g (Table 4.2) made up primarily of STX and dcSTX (Table 4.3).

Similarly, *Lophozozymus octodentatus* extracts exhibited simple toxin profiles including STX and dcSTX derivatives (Fig 4.7D; Table 4.3) and while toxin concentrations did not reach the levels observed in *A. floridus*, all were positive for STX-like activity by at least one method. Toxin levels and profiles obtained for *Thalamita stimpsoni* and *Pilumnus pulcher* by radio-receptor assay and LC-FD are provided in Table 4.2, Table 4.3 and Appendix 3 and comparisons between collections are shown in Fig 4.8.

Evidence of PSTs in Octopi

Each extract of *Octopus* (*Abdopus*) sp. 5 exhibited varying degrees of binding inhibition to [^{3}H] STX in the receptor assays and toxin concentrations ranged from 1.5- 494 µg STXeq /100 g tissue with five out of a total of 10 individuals within the regulatory limit for safe shellfish consumption (80 µg STXeq/100 g tissue; Fig 4.9B). Six additional octopi were dissected and organs and tissues extracted to identify tissue distribution of PST activity in these animals by radio-receptor assay. Toxin distribution in these animals was not limited to any particular organ or tissue as shown in Fig 4.10.

Table 4.3 Paralytic shellfish toxin composition of extracts from a variety of algae, molluscs and crustaceans collected from Cooke Point, Port Hedland. Toxin profiles were determined by HPLC analysis as described in "Methods".

Species	ID[#]	Trip[†]	Toxin Profile (mol %)					
			GTX 1	GTX 2	GTX 3	GTX 4	dcSTX	STX
Algae								
Sargassum sp. *	2	A	-	-	-	-	-	100
Jania sp. 2 *	3	A	11.2	49.5	34.4	4.9	-	-
Jania sp. 3 *	4[¶]	A	12.9	47.2	32.4	7.5	-	-
Molluscs								
Trochus hanleyanus	5	A	-	-	-	-	86	14
	6	A	18	-	-	3.2	24	54.8
	7	A	-	43	32	-	4.5	20.5
	8	A	-	21	18	-	-	61
Tectus pyramis	25	A	3.9	2.8	1	1.3	32	59
	26	A	3.4	-	-	2.3	3.3	73
	27	A	-	8.8	7.0	-	-	84.2
	28	A	-	-	-	-	10.8	89.2
Tridacna squamosa	9	A	-	-	-	-	100	-
	10	A	-	-	-	-	12	88
	11	A	-	22.5	6.7	-	-	70.8
Pinctada albina	13	A	-	-	-	-	-	100
sugilata	15	A	24	-	-	12	-	64
	16	A	-	-	-	-	93.8	6.2
Malleus(Malvufundus)	17	A	-	-	-	-	76	24
regula	18	A	-	-	-	-	-	100
	20	A	-	-	-	-	-	100
Saccostrea glomerata	21	A	-	-	-	-	97.8	2.2
	23[¶]	A	-	37	29	-	22	12
	24	A	-	-	-	-	89	11
Octopus (Abdopus)	29[§]	A	-	-	-	-	-	100
sp. 5	30	A	-	-	-	-	-	100
	31	A	-	-	-	-	-	100
	32	A	-	-	-	-	-	100
	79	B	-	-	-	-	3.7	96.3
	80	B	-	-	-	-	-	100
	81	B	-	-	-	-	-	100
	82	B	-	-	-	-	2.1	97.9
	84	B	-	-	-	-	1.1	98.9
Crustaceans								
Lophyzozymus	33	A	-	-	-	-	13	87
octodentatus	34	A	-	-	-	-	84.2	15.8
	35	A	-	-	-	-	76.4	23.6
	36	A	-	-	-	-	100	-
	49	B	-	-	-	-	3.4	96.6
	50[¶]	B	-	-	-	-	14.9	74.1
	51	B	-	-	-	-	83.6	16.4
	52	B	-	-	-	-	42.1	58
	53	B	-	3.2	1.6	-	-	95.2
	54	B	-	-	-	-	-	100
	55	B	-	-	-	-	10.9	89.1

[#] Extracts confirmed to contain PSTs by LC-FD analysis
* Pooled samples of at least 6 individuals
[†] Collections A and B as detailed in "Methods"
[¶] LC-FD spectra provided in Figure 4.9.
[§] LC-FD spectra provided in Figure 4.10.
- Below detection limits

Table 4.3 (*Continued*)

Species	ID[#]	Trip[†]	Toxin Profile (mol %)					
			GTX 1	GTX 2	GTX 3	GTX 4	dcSTX	STX
Atergatis floridus	37	A	-	-	-	-	9.4	90.6
	38	A	-	-	-	-	11.5	88.5
	39	A	-	-	-	-	100	-
	40	A	-	-	-	-	52.6	47.4
	57	B	-	-	-	-	34	66
	58	B	-	-	-	-	100	-
	59	B	3.2	-	-	1.4	88	7.4
	60	B	-	-	-	-	68.3	31.7
	61	B	-	-	-	-	58.4	41.6
	62	B	-	-	-	-	-	100
	63	B	-	-	-	-	20.1	35.6
	64	B	-	-	-	-	79.3	20.7
Thalamita stimpsoni	41	A	-	-	-	-	-	100
	43	A	-	-	-	-	2.1	97.9
	44	A	-	-	-	-	56	44
	65	B	6.7	21.3	15.2	2.3	-	54.5
	66	B	-	8.2	4.6	-	3.2	84
	67	B	-	3.5	1	-	-	95.5
	69	B	-	-	-	-	34.8	65.2
	72	B	-	-	-	-	100	-
Pilumnus pulcher	45	A	-	-	-	-	-	100
	46	A	-	-	-	-	1.8	98.2
	47	A	-	-	-	-	2.3	97.7
	48	A	-	-	-	-	-	100
	73	B	-	-	-	-	43	57
	75	B	-	4	3.4	-	-	92.6
	76	B	-	5.2	3	-	6.7	85.1
	77	B	-	38.8	27	-	2.2	32
	78	B	-	-	-	-	44	56

[#] Extracts confirmed to contain PSTs by LC-FD analysis
* Pooled samples of at least 6 individuals
[†] Collections A and B as detailed in "Methods"
[¶] LC-FD spectra provided in Figure 4.9.
[§] LC-FD spectra provided in Figure 4.10.
- Below detection limits

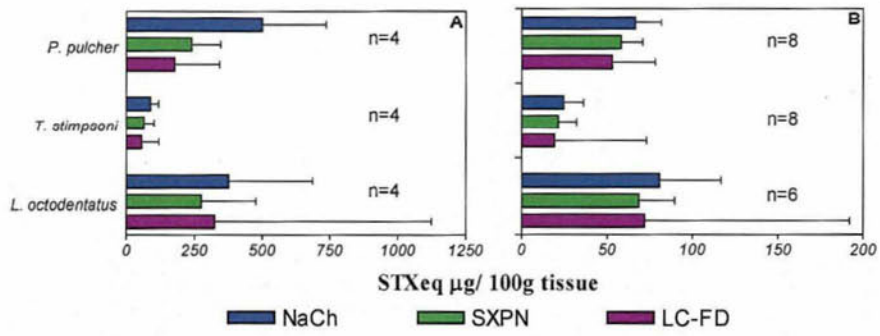

Figure 4.8 Total toxin concentration of crustacean extracts of *Pilumnus pulcher, Thalamita stimpsoni* and *Lophozozymus octodentatus* as calculated from sodium channel receptor assay (NaCh), centipede saxiphilin receptor assay (SXPN) and HPLC analysis (LC-FD). Saxitoxin equivalents (STXeq) from extracts of animals from field collection A (A) and B (B) were calculated as detailed in "Methods". Data are represented as the mean of toxin concentrations from a number of individuals of a single species. The top of each bar represents the minimum and the error bar extends to the maximum concentration observed for each species.

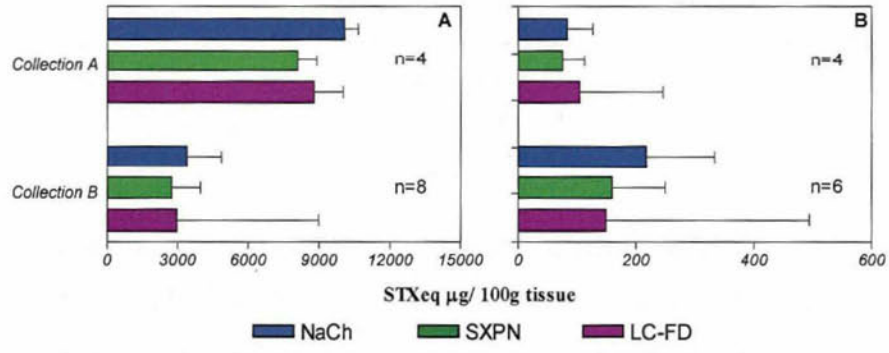

Figure 4.9 Total toxin concentration of extracts of *Atergatis floridus* (A) and *Octopus (Abdopus) Sp. 5* (B) calculated from sodium channel receptor assay (NaCh), centipede saxiphilin receptor assay (SXPN) and HPLC analysis (LC-FD). Saxitoxin equivalents (STXeq) from extracts of animals from field collection A and B were calculated as detailed in "Methods". Data are represented as the mean of toxin concentrations from a number of individuals from each species. The top of each bar represents the minimum and the error bar extends to the maximum concentration observed for each species.

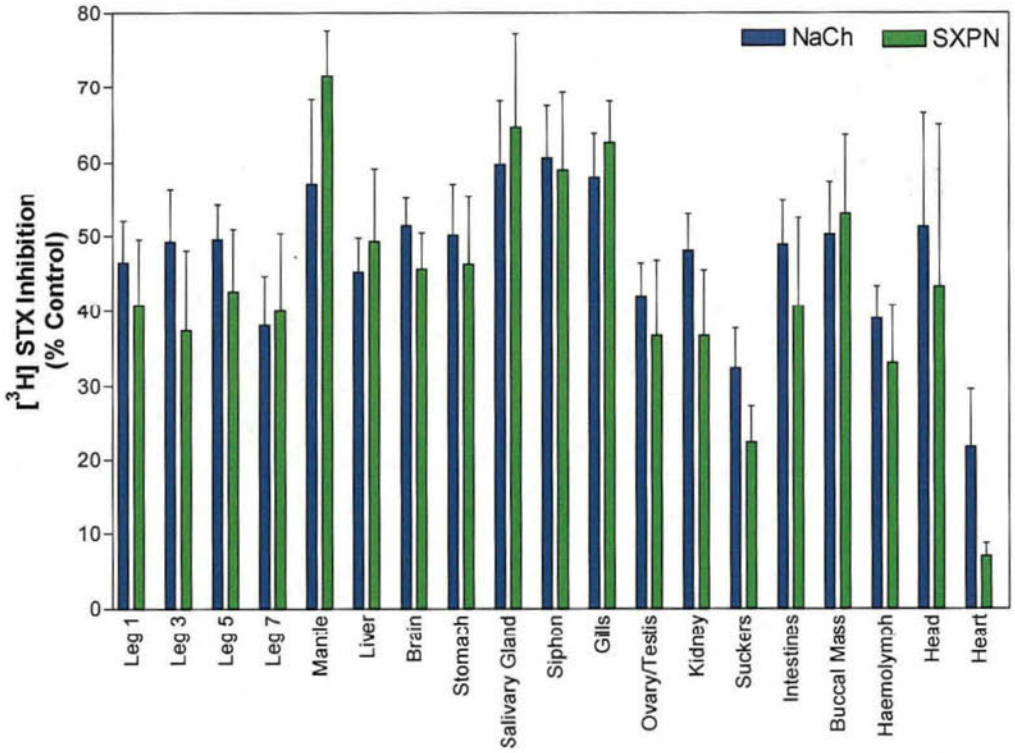

Figure 4.10 Tissue distribution of STX in *Octopus (Abdopus) sp. 5* as determined by sodium channel (NaCh) and centipede saxiphilin (SXPN) radio-receptor assays. Data are represented as the mean of [^{3}H] STX inhibition compared to 5μM STX control in tissue extracts of 6 individuals. The top of each bar represents the minimum and the error bar extends to the maximum concentration observed for each species.

LC-FD analysis of whole extracts indicated the presence of only a single toxin, STX, confirmed following elution of a peak at a retention similar to an authentic STX standard (Fig. 4.11A, B), and exhibiting fluorescence emission maxima at λ = 390 nm, consistent with PST. Further evidence for STX in the octopus tissue extracts was obtained following co-injection of samples with authentic STX. This spiking resulted in an increase in suspect peak height at the same retention time and magnitude observed for STX alone (Fig. 4.11C). Injection of octopus extracts without post-column oxidation resulted in the disappearance of the suspect STX peak (Fig. 4.11D). Most of the other peaks remained, including a small peak on the shoulder of STX, indicating that none of these were PST's.

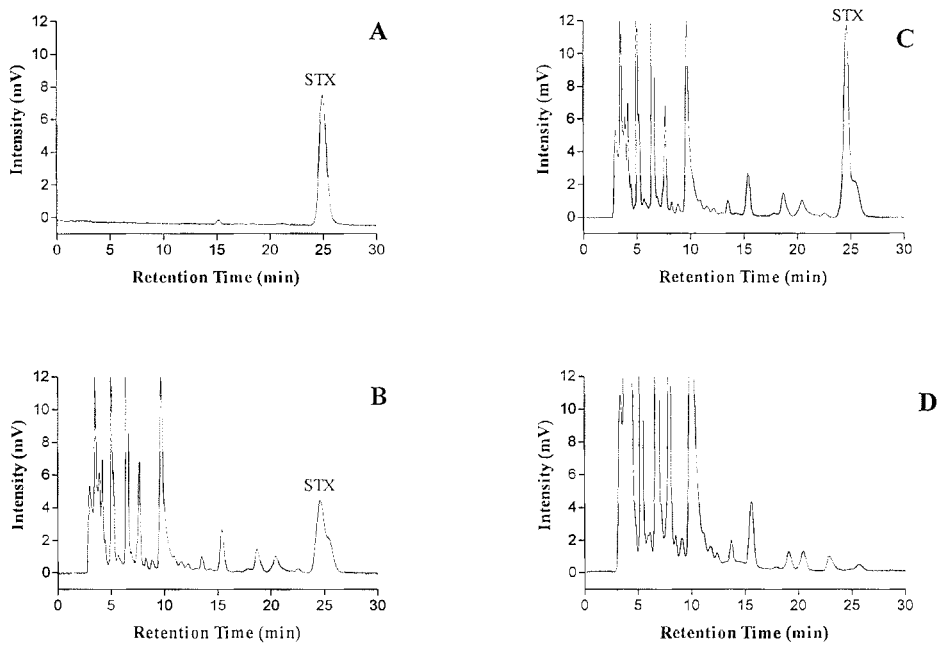

Figure 4.11 LC-FD chromatograms of calibrated standard STX injection (A) compared to a toxic extract of *Octopus (Abdopus) sp. 5.* (extract 29) showing peak retention (B), spiking of the extract with an authentic STX standard (C), and with no post-column oxidation (D).

4.3.2 LC-MS analysis of extracts from octopi

LC-MS data was obtained for octopi extracts exhibiting clearly identified STX peaks by LC-FD analysis. A strong peak was observed following SIM at m/z 300 in positive ion mode (extract 29; Fig. 4.12B), consistent with the expected pseudo-parent $[M+H]^+$ for STX (Fig. 4.12A). The background-subtracted daughter ion mass spectra of parent ions $[M+H]^+$ (m/z 300) for authentic STX and a toxic octopus extract, obtained by positive ion LC-MS/MS are shown in Fig. 4.13.

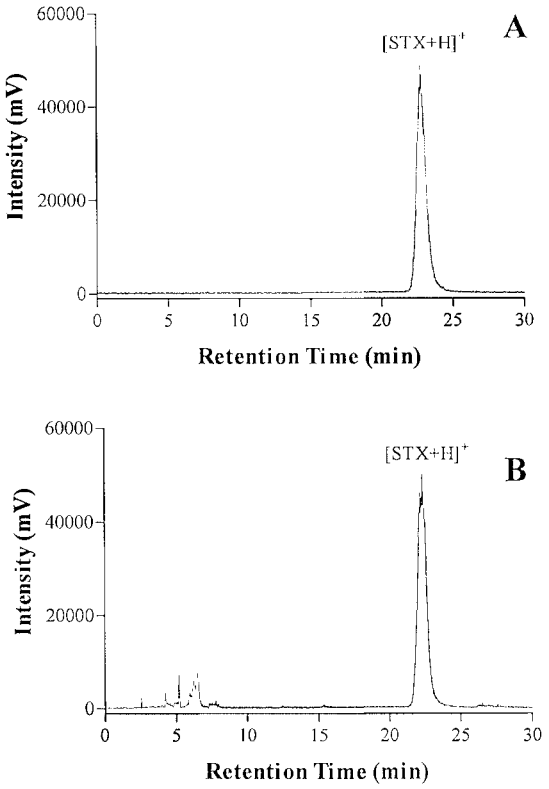

Figure 4.12 LC-MS chromatograms of an authentic STX standard (A) compared to extract of *Octopus (Abdopus) sp. 5.* (B; extract 29), all measured in single ion monitoring mode (m/z 300) on a triple quadrupole ion trap mass spectrometer.

MS^2 spectra reported correspond to the average of 5 individual spectra. The octopus extract exhibited fragment ions at m/z 282, m/z 265, m/z 240, m/z 239, m/z 204 and m/z 186 (Fig 4.13B), consistent with the fragmentation pattern observed for authentic STX (Fig. 4.13A), confirming the identity of STX in the octopus. Additional fragment ions were observed in the sample extract which correlate to previous findings (Quilliam et al., 1989). Suspected mass losses corresponding to the fragmentation of STX in the octopus samples are detailed in Table 4.4.

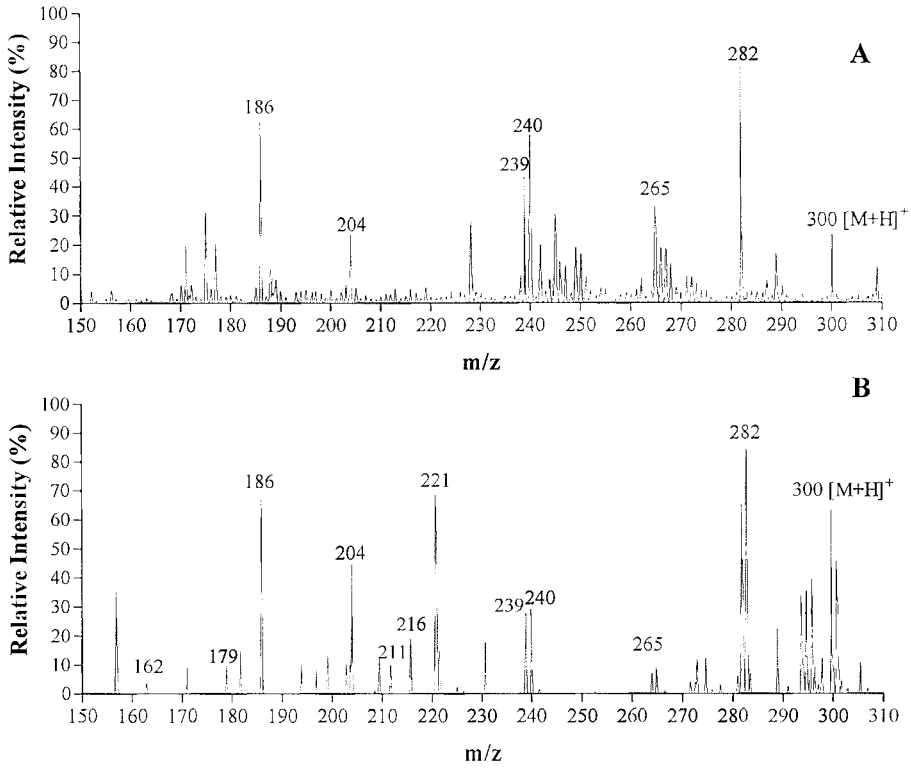

Figure 4.13 Daughter ion mass spectra for authentic STX (A), and an extract of *Octopus (Abdopus) sp. 5.* (B; extract 29) measured on a quadrupole ion trap mass spectrometer at m/z 100- 300 after fragmentation ofm /z 300 and with a mass accuracy of m/z 0.5.M S^2 spectra reported correspond to the average of 5 individual spectra.

Table 4.4 Observed mass losses from *Octopus (Abdopus) sp. 5* (extract 29) m/z 300 daughter ion mass spectrum. M/z in bold were also observed in the m/z 300 daughter ion mass spectrum of the authentic STX standard.

Observed m/z	Observed m/z loss	Suggested group loss
300	$(M+H)^+$	Parent Ion
282	-18	H_2O
265	-18, -17	$H_2O + NH_3$
240	-18, -42	$H_2O + CN_2H_2$
239	-61	CO_2NH_3
221	-18, -61	$H_2O + CO_2NH_3$
216	-42, -42	$CN_2H_2 + CN_2H_2$
211	-17, -72	$NH_3 + CH_2CH_2CH_2O_2$
204	-18, -61, -17	$H_2O + CO_2NH_3 + NH_3$
186	-42, -72	$CN_2H_2 + CH_2CH_2CH_2O_2$
179	-18, -61, -42	$H_2O + CO_2NH_3 + CN_2H_2$
162	-18, -42, -17, -61	$H_2O + CN_2H_2 + NH_3 + CO_2NH_3$

4.3.2 Occurrence and characterisation of STXBPs from Port Hedland

80 individuals from 11 species including gastropods, bivalves, octopi and crabs were extracted and screened for STX binding protein activity during the course of this investigation. STX binding activity was only identified in 9 protein extracts from *Lophozozymus octodentatus* from total of 12 individuals tested. Levels of detectable STX binding activity (pmol/ g tissue) ranged from 0.2-88.4 pmol/ g tissue and showed an inverse relationship to PST concentrations on an individual basis (see Fig 4.14). Saxitoxin binding activity from *L. octodentatus* could not be displaced by 5 μM TTX or Fe^{3+}.

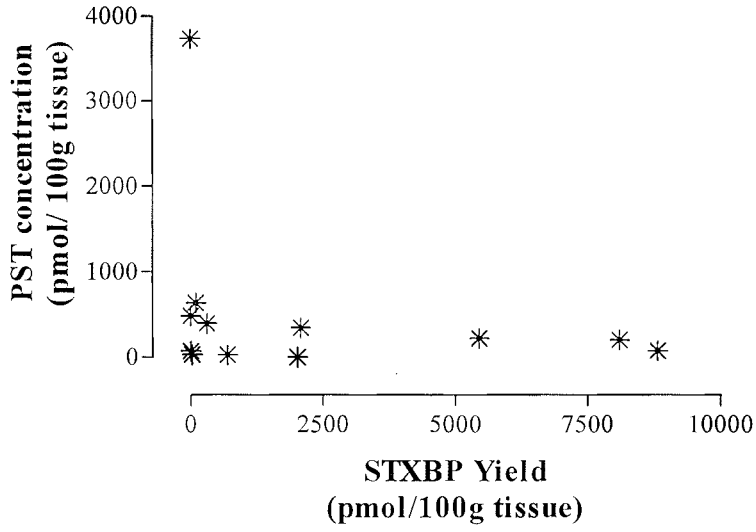

Figure 4.14 Relationship between STXBP and PST levels in the Xanthid crab *Lophozozymus octodentatus* collected from Port Hedland. Toxin concentrations were quantified by LC-FD as detailed in "Methods" and were converted from μg STX equivalents/ 100g tissue to pmol/ 100g tissue for graphic. STXBP yields are the mean of 3 MCE protein binding experiments performed in duplicate.

All extracts of *L. octodentatus* from collection B were pooled for further pharmacological characterisation. Binding site titration of pooled *L. octodentatus* extract revealed that increasing the amount of extract (total protein) in the assay caused a concomitant linear increase in the amount of receptor bound [^{3}H] STX (Fig 4.15A). Saturation binding experiments revealed that in all active extracts [^{3}H] STX binding was saturable and contained a component of linear non-specific binding (Fig 4.15B).

Aliquots of pooled *L. octodentatus* extracts was boiled and digested in trypsin resulting in a complete loss of pre-treatment activity. Ultrafiltration size estimation was also performed for *L. octodentatus* extracts and activity was recovered at molecular weights between 50 - 100 kDa, with some activity remaining at sizes between 100 – 200 kDa.

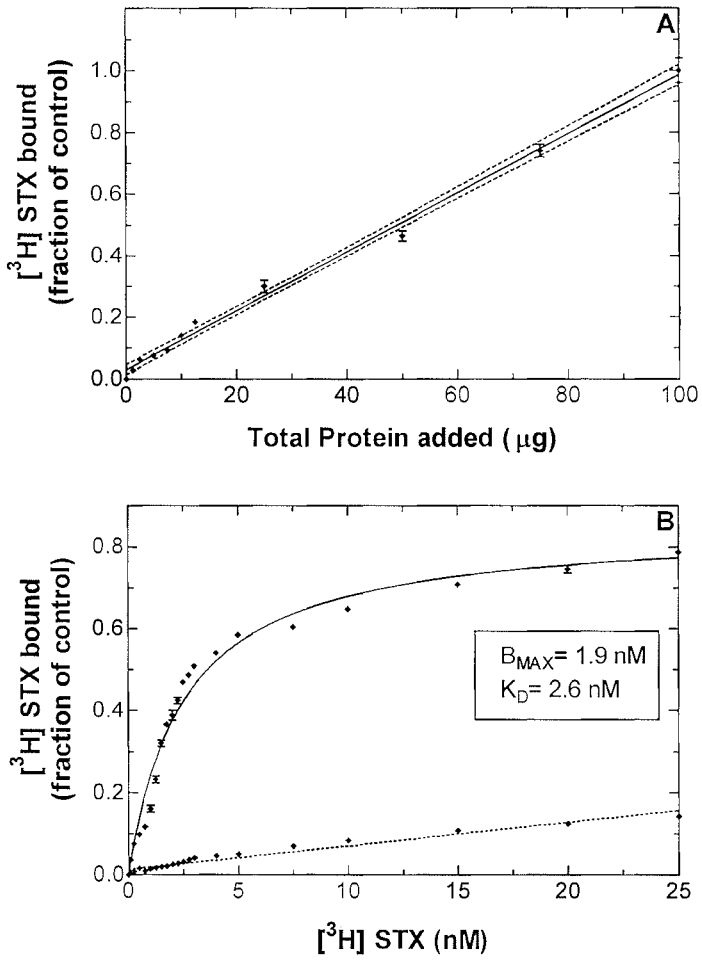

Figure 4.15 Binding site titration (A) and saturation binding curve (B) in protein extracts from *Lophozozymus octodentatus* collected from Cooke Point, Port Hedland. Data representm ean ±S EM from 3 experiments performed in duplicate. In some instances symbols obscure the error bars. K_d and B_{max} were approximated to the nearest decimal and calculated by non-linear regression using one site model. Further interpretation is provided in "Results".

4.4 Discussion

4.4.1 PST occurrence & potential health implications

Cooke Point, located in Port Hedland on the north western coast of Australia was chosen as a unique study site for the examination of PSP producers, vectors and the occurrence of saxitoxin binding proteins, based on previous findings of high PST prevalence in 19 of 20 individuals from six crustacean species collected from the area (Negri and Llewellyn, 1998). In the present study, 84 animals representing 15 species (4 algae, 2 gastropods, 4 bivalves, 1 octopi, 4 crabs; Table 4.1) were collected, extracted and analysed by two well published radio-receptor assays (NaCh; SXPN; (Llewellyn and Doyle, 2001; Llewellyn et al., 1998) and LC-FD analysis making this study the most extensive examination of the occurrence and toxicity of PSTs in intertidal animals from Port Hedland to date. The three methods used to determine STX concentration (NaCh, SXPN, LC-FD) agreed closely with each other, exhibiting correlations $0.98 < R^2 > 1.0$ for all comparisons, deemed significant ($p < 0.05$) in all cases (Fig 4.6). The high correlations observed for the data set are very high, which is probably due to the fact that the data set clustered at high and low concentrations, with only a few species found at concentrations in between, and as such may not be a true indicator of method comparison in this instance.

PSTs were detected in 73 of 84 individuals collected at concentrations ranging from 0.6 µg STXeq/100 g tissue in algae to as high as 10,029 µg STXeq/100 g tissue in specimens of *Atergatis floridus*, as determined by LC-FD (Table 4.2; Appendix 3). 38 extracts (LC-FD data) exceeded the regulatory level (RL) of 80 µg STXeq/100 g wet weight tissue for safe human consumption of shellfish, which has been adopted by most western countries (Bricelj and Shumway, 1998). However, the majority of extracts were calculated at STXeq levels within the safe limit.

4.4.2 Bioaccumulation of PSTs in benthic food web

All species of algae examined in this report were identified with PST activity by radio-receptor assay (NaCh; SXPN) including the brown algae *Stoechospermum sp.* and *Sargassum sp.* and 2 rhodophytes from the genus, *Jania* (*Jania sp.2; Jania sp.3*). The presence of known STX derivatives in extracts of *Stoechospermum sp.*

could not be confirmed by LC-FD but a trace amount of STX (0.6 μg/100 g; Table 4.2) was identified in *Sargassum sp.* (Table 4.3). Four filter-feeding bivalves, including *Tridacna squamosa, Pinctada albina sugilata, Saccostrea glomerata* and *Malleus (Malvufundus) regula,* from Port Hedland field collection A, were added to the list of known PST vectors for the first time during the course of this investigation, although several closely related species have been reported to harbour PSTs (Bricelj and Shumway, 1998; Harada et al., 1982). Saxitoxin in the tissues of *Octopus (Abdopus)* sp. 5, however, is the first reported incidence in this family of predatory molluscs and consolidates the presumed pathway in marine food webs (see Fig 4.1).

It is common for tropical intertidal zones to harbour multiple species of PST-contaminated crabs and molluscs, with one early report indicating that PST's may be present throughout these food webs (Oshima et al., 1984a). In that study, organisms including crabs and molluscs were found to contain significant levels of PST and the primary source was reported to be the red algae *Jania* sp. (Kotaki et al., 1983; Oshima et al., 1984a). In the present study, two species of *Jania* were found to contain sulfated carbamate STX derivatives: GTX 1, GTX 2, GTX 3 and GTX 4 (Fig 4.7c; Table 4.3) and were calculated at 16 μg STXeq/100 g and 23 μg STXeq/ 100 g for *Jania* sp 2. and *Jania* sp.3., respectively (Table 4.2). Similar toxin profiles have been reported in *Jania* sp.1. and shown to contain GTX1, GTX 2 and GTX 3 at a ratio of 73: 31: 6 (Oshima et al., 1984a).

Simple toxin profiles consisting of dcSTX and STX in most animals (Table 4.3) corroborate the food web theory of PSP bioaccumulation in marine benthic ecosystems. Although the causative source of PSTs in these animals is unclear, it is probable that source organisms may consist of more complex toxin profiles. Most shellfish contaminated with PSTs contain less C-toxins and more carbamate toxins that the causative dinoflagellates, due to toxin transformations within the animals (Oshima et al., 1990). This phenomenon is due to enzymatic reductive cleavage such as desulfation and dehydroxylation and hydrolysis (decarbomoylation and desulfation) of various C-toxins and GTXs (Jones and Negri, 1997; Kotaki et al., 1985; Sullivan and Iwaoka, 1983).

4.4.3 Saxitoxin in octopi

A total of 16 specimens of *Octopus (Abdopus)* sp. 5 extracts of tentacle (n=4; Collection A), whole animal (n=6; Collection B) and a variety of tissues and organs (n=6; Collection B) were examined during this study, 14 of which contained STX. Two of the extracts from collection A were within the RL for safe shellfish consumption (80 µg STXeq/100 g tissue). One sample was contaminated with between 72 – 103 µg STXeq/100 g tissue and another contained 2 – 3 times limit in both assay formats and by LC-FD (Fig 4.9b). Collection B yielded significantly higher toxin concentrations ($p<0.05$ ANOVA; Fig. 4.9b) in octopi, with three out of six animals exceeding the RL and reaching levels between 494 – 695 µg STXeq/100 g tissue in one instance (extract 79).

In collection A, the tentacles of *Octopus (Abdopus)* sp. 5 were targeted for analysis as these have the least tissue complexity and are the organs most likely to be consumed in octopi of this size. STX was identified as the major contributing toxin in these extracts by LC-FD (Fig 4.11) and structural confirmation by LC-MS analysis (Fig 4.12) and parent ion (STX $[M+H]^+$) fragmentation (Fig 4.13; Table 4.4) unequivocally consolidated this finding. LC-FD analysis of animals collected in 2002 revealed the additional, yet minimal contribution of dcSTX in three extracts but this could not be confirmed by LC-MS due to sample complexity, low concentration of dcSTX and detection limits of the technique.

Examination of the tissue distribution of STX in *Octopus (Abdopus)* sp. 5 involved the isolation and extraction of the stomach, intestine, kidney, liver, ovary/testis, brain (including nerve bundles), heart, haemolymph, secondary salivary gland, buccal mass (including anterior mouthparts/beak and primary salivary gland), gills, and tissue from mantle, siphon, head, legs and suckers. All tissue extracts were then analysed by radio-receptor assay, revealing inhibition of $[^3H]$ STX binding in all extracts with highest levels recorded in extracts of salivary gland, siphon, gills, head and buccal mass. This distribution implies a more widespread distribution of the STX than is reported for TTX in the blue-ringed Octopodidae relatives (*Hapalochlaena maculosa, H. lunulata, and H.*

fasciata) (Edgar, 2000). In *Hapalochlaena spp.* the neurotoxin TTX resides almost entirely within the salivary glands (Lane and Sutherland, 1967).

In Australia, octopus represent a small but significant wild-catch fishery with over 700 metric tonnes (mT) caught in 2001-2002 for processing and food production, worth over US$2.4 million (ABARE, 2003). Octopus fishing in Western Australia is approximately 16% of total Australian catch (117.8 mT landed weight 2001-2002) targeting *Octopus tetricus* as well as several other undescribed octopus species (Cliff, 2003). The discovery of STX in *Octopus (Abdopus)* Sp.5 highlights the need for greater public awareness of the risks associated with consumption of previously unrecognised vectors of paralytic shellfish poisoning (PSP) in the region.

4.4.4 Environmental sources of PSTs

In 2002, total maritime trade in Port Hedland was in the order of 72.4 million tonnes, making it the third largest port in Australia and the ninth largest bulk commodity port in the world (PHPA, 2003). The majority of international trade through the port comes mainly from South East Asia including Japan, South Korea, Singapore, China, Taiwan, Indonesia, Malaysia, Phillipines, Irian Jaya, Vietnam and Thailand (PHPA, 2003), which have all been shown to have a high incidence of PSP. The threat of introduction of pest species such as PST-producing dinoflagellates to Port Hedland via domestic and international ballast water has been well recognised and ongoing management and monitoring programs are legislated by the Commonwealth (PHPA, 2003). *Alexandrium catenella* was found, isolated and sequenced from Port Hedland Ballast water (Hallegraeff, 1998) and another survey identified a *Gymnodinium* species of unknown toxicity (Hewitt et al., 1999) however there is little conclusive evidence of PSP producing dinoflagellates in surrounding waters (Hallegraeff, 1993; Hallegraeff and Jeffrey, 1984; Hewitt et al., 1999).

There are four possible contributing sources of PSTs in Port Hedland including: 1) macro algae such as *Jania sp.;* 2) epi-plants on macro-algae; 3) dinoflagellates; and 4) bacteria.

In basic terms macro algae, like phytoplankton, represent the lowest level in marine benthic food webs and while few species have been implicated in PSP, they are good contenders for primary production in Port Hedland. All four species of algae examined were found to contain STX activity using bioassays and this was confirmed by LC-FD in three species. One clear contributing source of PSTs in Port Hedland was identified in the red algae *Jania sp. 2* and *Jania sp. 3* , a genus which has been previously implicated as the primary source of PSTs in tropical waters (Oshima et al., 1984a). Only GTX 1-4 were identified in *Jania* supporting reports of GTX1-3 in these rhodophytes (Kotaki et al., 1983; Oshima et al., 1984a; Sakamoto et al., 1992). Future collections of *Jania* are planned to enable structural confirmation of PSTs by LC-MS and examination of seasonal and geographical variation in toxicity.

The finding of STX in *Sargassum sp.* is fascinating and is a good candidate for a more extensive analysis. Interestingly, cold water strains of the marine dinoflagellate *Prorocentrum lima* have been found to be distributed on the surface of a related algae *Sargassum confusum,* and shown to produce the diarrhetic shellfish poison (DSP), okadaic acid (OA) (Koike et al., 1998). Other members of the *Prorocentrum* genus have also been reported to produce toxins including OA derivatives (*P. belizeanum),* yessotoxins (*P. reticulatum*), haemolytic toxins (*P. mexicanum*) and other unidentified neurotoxins (*P. borbonicum*) (Lee et al., 1989; Murakami et al., 1982; Nagajima et al., 1981; Satake et al., 1997; Ten-Hage et al., 2000). Based on this evidence it is plausible that some newly toxic strains of *Prorocentrum spp.* such as *P. micans,* recently discovered in a phytoplankton survey of Port Hedland (PHPA, 2003) may be associated with *Sargassum sp.* and even capable of producing PSTs or other sodium channel inhibitors.

Evidence of PSTs in brown and red algae from Port Hedland is an interesting finding but does not fully explain the levels of toxins observed in some species. Back calculations of toxicity in *A. floridus* for instance indicated that the crab would need to eat over half a tonne of *Sargassum* to reach the levels of toxicity observed for this species (Table 4.5). In addition, PSTs were observed in filter feeders such as oysters and mussels which clearly could not have consumed *Jania* or *Sargassum.* The most logical explanation, supported by known toxicity and

rates of bioaccumulation (Table 4.5), suggest that a dinoflagellate source contributes to toxicity to benthic animals in Port Hedland.

Bivalve toxin profiles determined during this study (Table 4.3) consisted of varying levels of STX, dcSTX and GTXs 1-4, which provide the only traceable marker for prediction of a likely dinoflagellate source. Prevalent sub-tropical dinoflagellates of the Asia-Pacific region include *Pyrodinium bahamense* var *compressa*, *Alexandrium minutum* and *Gymnodinium catenatum*.

Table 4.5 Calculations of gross amount of PST source that a specimen of *Atergatis floridus* would be required to consume to acquire concentrations of 11,336 µg/100g tissue as observed from Port Hedland during this study.[§]

Source of PSTs	Recorded Toxicity[†]		Amount to consume[‡]	Time to reach toxicity[¶]	Reference*
	MU/g	STXeq µg/g			
Dinoflagellates					
Gymnodinium catenatum	1300	234	14.5 g	2.4 days	(Oshima et al., 1987)
Alexandrium minutum	1333	240	14.2 g	2.4 days	(Hallegraeff et al., 1988)
Algae					
Jania sp 1	1.5	0.27	13 kg	6 yr	(Oshima et al., 1984a)
Jania sp 3	1.3	0.23	15 kg	7 yr	Table 4.3
Sargassum	0.03	<0.01	567 kg	259 yr	Table 4.3
Cyanobacteria					
Anabaena circinalis	3167	570	6 g	< 1 day	(Jones and Negri, 1997)

[§] *Atergatis floridus* collected from Port Hedland (extract 38). Toxicity measured by Na-channel assay

[†] Conversions based on 1 MU= 0.18µg STX (Schantz et al 1957) wet weight.

[‡] Approximate values based on 30g specimen assuming 100% absorption and 0% elimination

[¶] Approximate values calculated assuming crab consumes 20% body weight in algae per day i.e. 6g/day

* References from which toxicity levels were derived

Of these species, *A. minutum* is the most likely candidate with toxin profiles consisting of GTX 1-4 and STX (Table 4.6). Toxin profiles observed from *P. bahamense* var *compressa* may explain the prevalence of STX and dcSTX observed in Port Hedland extracts however the complete lack of neoSTX, GTX 5 and GTX 6 is contradictory. *G. catenatum* is clearly an unlikely source with toxin profiles dominated by C-toxin derivatives which were not observed in any

specimens from Cooke Point. Lack of reports of dinoflagellate blooms may indicate that the dinoflagellate may be present as resting cysts which have been shown to reach toxicities ten times greater than their motile stage counterparts (Dale *et al.* 1978).

Table 4.6 Toxin profiles of 3 common PST-producing dinoflagellates from Asia Pacific region.

Dinoflagellate species	Toxin Profile	Reference
Pyrodinium bahamense var *compressor*	neoSTX > STX > dcSTX = GTX5 >GTX6	(Oshima et al., 1984a)
Alexandrium minutum	GTX1 > GTX 4 > STX > GTX 2 > GTX 3	(Negri et al., 2003a)
Gymnodinium catenatum	C4 > C3 > GTX 5 > GTX 6 > C1> C2	(Negri et al., 2003a)

Lastly, PST production in algae, molluscs and crustaceans may be associated with or facilitated by marine bacteria. Evidence for PST production by bacteria has been reported, but is controversial with few studies providing clear structural verification of PST production (for review see Gallacher and Smith, 1999). However, anecdotal observations of consistent PST toxicity of crustaceans such as *A. floridus,* collected from coral reefs around the globe (Arakawa et al., 1995; Raj et al., 1983; Tsai et al., 1997) support this hypothesis. Similarly, there are many reports whereby the causative micro-algal profile does not correspond to the toxin profile observed in contaminated shellfish (Asakawa et al., 1995; Cembella et al., 1993). Evidently, enzymatic activity of shellfish extracts facilitate interconversion of PSTs (Cembella et al., 1993; Sullivan et al., 1983) as mentioned in 4.2.2 but it has also been suggested that bacteria such as *Vibrio* and *Pseudomonas* spp. isolated from shellfish may convert PSTs by reductive elimination (Kotaki et al., 1983; Smith et al., 2001) which may explain the pre-dominance of carbamate derivatives in samples analysed in this study.

4.4.5 Occurrence of STXBPs in PST "Hot Spot"

STX binding activity was examined in 80 invertebrate animals including gastropods, bivalves, octopi and crabs collected from Port Hedland. Novel STX specific binding activity was confirmed in nine out of 12 individuals from the Xanthid, *L. octodentatus.* The identity of this component is reminiscent of the

transferrin homolog, bullfrog saxiphilin, based on: 1) specific, high affinity binding to STX; 2) hydrophilic; 3) inability to bind TTX; (4) estimated size range between 50- 100 kDa; and 5) degradation of activity following boiling and trypsin digest, as discussed in Chapter 3.

Complete purification sequencing and functional expression is required to fully elucidate the relationship and identities of this STX binding component. At an individual level, the occurrence of STX binding activity in *L. octodentatus* appeared to have an inverse relationship to PST concentrations (see Fig 4.14) with highly toxic animals having the least STX binding activity. This phenomenon needs to be explored more thoroughly but does suggest that either 1) the presence of STXBPs is not related to PST bioaccumulation; or 2) STXBPs may promote elimination of PSTs rather than protection. The extremely high prevalence of PSTs in Port Hedland may explain individuals in toxin concentrations were highest and may have been beyond the binding capacity of the protein. Further investigation is required to elucidate the relevance of minimal co-occurrence in this and other species.

In other benthic animals from Port Hedland that were identified with PSTs in tissue extracts, hydrophilic STX binding activity was consistently absent and in these species, survival from STX exposure did not appear to be dependent upon the existence of STXBPs in other benthic animals. It should be noted that animals from collection A has been stored at -20 °C for an extended period of time prior to protein and toxin extraction and so the possibility of protein degradation during this period can not be excluded however two *L. octodentatus* specimens from collection A (34, 35) showed greater specific activity than all animals from the more recent collection B ($P>0.05$; Fig 4.14).

If STXBPs do not have a protective role in animals from the marine environment, it is highly likely that an endogenous ligand of similar structure to STX must exist within the organisms which express it, a notion that was confirmed by the inhibition of STX by MeOH extracts of the terrestrial amphibian *Bufo marinus* in the previous chapter (Chapter 3). Further characterisation of this component will be examined in Chapter 5.

4.5 Conclusions

This study confirmed 3 new sources of PSTs in benthic food web of Port Hedland including *Sargassum sp. Jania sp.2* and *Jania sp.3*, however these did not explain the extreme levels of STX observed in some species. A number of new vectors were also identified from the bivalves *Tridacna squamosa, Pinctada albina sugilata,Saccos trea glomerata* and *Malleus (Malvufundus) regula* examined from the region and resulted in the first report of STX in an octopi *Octopus (Abdopus) sp. 5*. A novel STXBP was identified in the Xanthid, *Lophozozymus octodentatus* but the lack of widespread STXBPs in the intertidal STX "hot spot" does not conclusively support the toxin defence hypothesis as a likely biological role of STXBPs. Further investigation is required to fully determine the relevance of co-occurrence of STX and STXBPs in this species.

4.6 Acknowledgements

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CHAPTER 5

Characterisation, Purification and Partial Sequencing of a Saxitoxin Binding Protein from *Bufo marinus*

5.1 Introduction

Bufo marinus, commonly known as the giant marine or cane toad (see Fig. 5.1) was first introduced into the Gordonvale region of Queensland, Australia in June, 1935. The introduction was based on evidence from earlier releases into Puerto Rico and Hawaii where they were successfully established as a biological control to the sugar cane beetle *Lepidoderma albohirtum* (Easteal, 1981). Successive introductions to the major sugar cane growing areas in regional Queensland followed with *B. marinus* offspring released in Cairns, Ingham, Giru, Ayr, Mackay and Bundaberg throughout the late 1930s (Easteal, 1985). The sub-tropical and tropical habitats in these regions provided the perfect environment and a plethora of invertebrate food sources and this ecological advantage was pivotal to the success of the toad, with populations now extending from the surrounding areas of Darwin in the Northern Territory to northern New South Wales (Aldhous, 2004).

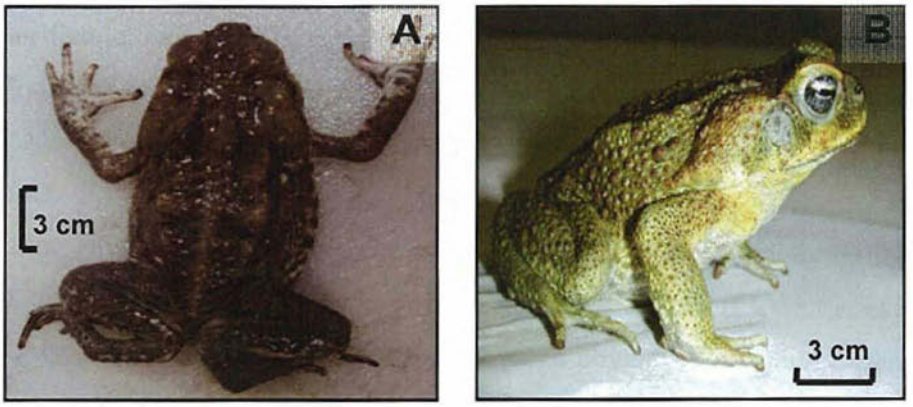

Figure 5.1 Female (A) and male (B) adult specimens of the cane toad, *Bufo marinus*.

With no natural predators in Australia, the success of cane toad populations has been at great cost to terrestrial ecosystems and has been associated with the demise in populations of native frogs and other native insectivores (Aldhous, 2004). Due to a fierce appetite and non-specific food preference, toad populations have also flourished in urban areas and numerous poisonings of domestic animals have been reported (Aldhous, 2004). In some eastern cultures, toad venom, located in the parotid gland, is used as an aphrodisiac and its recreational use has resulted in several human casualties (Gowda et al., 2003). Toad venom (bufotoxin) is comprised of a cocktail which includes cardiac glycosides (Radford et al., 1986) and steroids similar to digoxin. Bufotoxin poisoning causes gastrointestinal effects including nausea and vomiting due to effects on the sympathetic nervous system and cardiac effects due to atrioventricular conduction blockage such as brachycardia, ventricular tachycardia, fibrillation and sudden death (Bagrov et al., 1993; Gowda et al., 2003).

5.1.1 Aims

Despite its unpleasant appearance and reputation, the prevalence and availability of *B. marinus* in Australia provided a useful model for the examination of saxitoxin binding proteins (STXBPs), an activity first recognised by Llewellyn et al., (1997) during a phylogenetic survey of STX binding activity. The use of *B. marinus* as a model system was a logical first step based on the previous identification of saxiphilin (SXPN) in the North American bullfrog, *Rana catesbeiana,* which remains the only hydrophilic STX specific receptor that has been purified, cloned and functionally expressed to date (Krishnan et al., 2001; Li and Moczydlowski, 1991; Morabito et al., 1995; Morabito and Moczydlowski, 1994; Morabito and Moczydlowski, 1995). As a member of the phylum Amphibia, *B. marinus* STXBP may have some similarity to *R. catesbeiana* saxiphilin. However, other than the observed STX binding activity in both species, no additional similarities have been identified to date (Llewellyn et al., 1997). Also, while *B. marinus* belongs to the same phylum it is from a different class, family and genus to the Ranid frogs and as such it may be expected to carry many different characteristics, particularly at a molecular level. Differences between toad STXBP and bullfrog SXPN cannot be determined without purification, sequencing and functional expression, some of which will be

examined during this chapter. Bullfrog SXPN was reported to have almost ubiquitous distribution within the animal tissues, possibly due to its mobile presence within plasma, and across all life stages (Mahar et al., 1991). If toad STXBP is related to bullfrog SXPN it would be expected that the tissue distribution and presence at different life stages would be similar. Given the vastly different geographical regions from which each animal has originated, the biological requirement for the STX binding proteins may differ. In contrast, the influence of environmental parameters such as temperature on SXPN or STXBP yields in STXBP-containing animals has never been investigated. Factors such as these may provide clues to the biological role of this unique suite of receptors, particularly as STX binding activity has only been identified in ectothermic organisms. The biological function of SXPN and STXBPs remains a mystery and while the possibility of a defensive role against STX intoxication has been extensively examined during the course of this study (see Chapter 4) preliminary evidence of an endogenous ligand in *B. marinus* suggests an alternative role for these proteins (see Chapter 3). Further investigation of this activity may elucidate the relevance and possible identity of this ligand.

The specific aims of this Chapter were to:

- Examine the distribution of STX binding activity in *B. marinus* tissues
- Isolate the endogenous STXBP ligand identified in MeOH extracts of *B. marinus*
- Determine whether STX binding activity is present across all life stages of *B. marinus*
- Purify *B. marinus* STXBP from plasma by chromatography and electrophoresis
- Retrieve peptide sequence from purified STXBP digests and design degenerate nucleotide primers for degenerate polymerase chain reactions (PCR)
- Amplify STXBP nucleotide fragments by degenerate PCR using cDNA from *B. marinus* liver then clone inserts into a pGEM®-T Easy vector
- Determine amino acid sequence homology to SXPN, transferrin (TF) and any other proteins from public databases.

5.2 Materials and Methods

5.2.1 Reagents and equipment

All general reagents and chemicals were purchased from Sigma (Castle Hill, NSW) unless otherwise stated and water was deionised (~18MΩ) with a Millipore MilliQ system (North Ryde, NSW). Ultrafree MC Biomax molecular weight filter units, mixed cellulose syringe filters (0.45 μM), and all 96-well Multiscreen™ microplates (MCE) were purchased from Millipore (North Ryde, NSW). Optiphase Supermix scintillation fluid and 96-well MicroBeta sample plates were supplied by Perkin Elmer (Knoxfield, VIC). Complete protease inhibitor cocktail tablets (cØmplete protease inhibitors) were purchased from Roche (Dee Why, NSW). Tritiated STX ([^{3}H] STX) was purchased from Amersham Pharmacia Biotech (UK). Saxitoxin dihydrochloride (STXdiHCl) was purchased from the National Research Council of Canada, Institute for Marine Biosciences, Certified Reference Materials Program (Halifax, NS, Canada).

Liquid nitrogen was purchased from BOC Gases (Townsville, Australia) and was at least 99.99% pure. HPLC grade MeOH was from Mallinckrodt (Biolab Scientific, Clayton, SA). Liquid chromatography-Fluorescence Detection (LC-FD) reagents and PST analytical standards were obtained as previously described (see Chapter 4). Protein electrophoresis equipment, chemicals and consumables were supplied by BioRad (Reagents Park, NSW) and purification consumables were purchased from Amersham (Superdex 200 HR; Superdex G50, Q-Sepharose). Ambion micro poly (A) purist kit was purchased via GeneWorks (Thebarton, SA). BD Powerscript reverse transcriptase and 5 × First Strand Buffer was purchased from BD (North Ryde, NSW) and additional PCR reagents and kits were purchased from QIAGEN (Clifton Hill, VIC) unless otherwise stated. pGEM®-T Easy Vector System II containing JM109 high efficiency competent cells was purchased from Promega (Annandale, NSW). All oligonucleotides were synthesised and desalted by Sigma-Genosys (Castle Hill, NSW). DYEnamic ET reaction mix was from Amersham Biosciences.

5.2.2 Animal collection

Bufo marinus specimens were hand collected from urban locations within Townsville, QLD. A total of 40 adults were collected during the course of this thesis between 1st May 2002- 30th April 2004. 10 juvenile toads (2-15 g in weight) and 20 toadlets (<1 g; first land metamorph) were also hand collected while 50 toad-poles (from three metamorphic stages) were collected by net from shallow stagnant water and maintained in glass holding tanks containing aerated de-chlorinated tap water at 25 °C. Adult and juvenile forms were sacrificed according to the guidelines of JCU Animal Ethics Sub-committee (Approval A745; see Chapter 2). To minimise animal usage, in most instances a single animal was used for more than one experiment (e.g. plasma collection for purification and tissue/ organ harvest for extraction). Toad-poles were anaesthetised by cooling in iced water for 10 min. then sacrificed by freezing at -20 °C, overnight.

5.2.3 Tissue distribution

Five adult cane toads were dissected and tissues including liver, heart, kidney, brain, lung skin, blood, heart, fat bodies, oocyte, stomach, large intestine, small intestine, venom glands and skeletal muscle removed and pooled for protein extraction. Freshly dissected tissue was rinsed with cold phosphate buffered saline (PBS, pH 7.4), frozen in $N_{2\ (l)}$, then ground to a powder by mortar and pestle. Following reconstitution in 20 mM HEPES-NaOH (pH 7.4) containing a cocktail of protease inhibitors (cØmplete, Roche), extracts were homogenised on ice using a PRO250 tissue disruptor for 3 × 30 sec. on highest setting then centrifuged for 20 min. at 15000 × g, 4 °C (Hermle Z323K refrigerated centrifuge) to remove particulate matter and membrane materials containing sodium channels. Supernatants were then filtered through 0.2 μM cellulose acetate filters (Millipore) and stored at -80 °C until assay. Activity was determined using the MCE protein binding assay with 2 nM [^{3}H] STX (see Chapter 2).

5.2.4 Endogenous Ligands

Selected tissues and organs (as described in section 5.2.3) from five adult *B. marinus* specimens were pooled and extracted with methanol (2 mL/ g based on evidence of [^{3}H] STX binding competition for *B. marinus* STXBP extract in MeOH extracts of toad viscera (see Chapter 3). Extractions were prepared by

homogenisation of minced tissue in MeOH for three 30 sec. bursts with a PRO250 tissue disruptor (10 mm head; highest setting), followed by sonication for 80 min. (Transtek Bath Sonicator, Selby Biolab). Homogenised extracts were left to steep in sealed containers for two days at ~25 °C, after which they were centrifuged at 10,000 × g for 30 min. to pellet particulates. Supernatants were filtered (0.22 µM nylon) and transferred in to pre-weighed scintillation vials while pelleted material was re-extracted twice as described. Filtered supernatants from successive extractions were pooled and dried *in vacuo*, then reconstituted at 10 mg/ mL in MeOH (HPLC grade) and assayed against *B. marinus* STXBP extract (50 µg total protein) to identify any displacement of [^{3}H] STX.

Pooled *B. marinus* viscera (200 g) from an additional five toads was extracted with MeOH (400 mL) as described, then filtered (0.22 µM nylon) and concentrated *in vacuo*. The filtered MeOH extract was then subjected to C18 Solid Phase Extraction (SPE) chromatography (10% gradient elution from 10% MeOH/ H_2O to 100% MeOH/ H_2O). Active MeOH/ H_2O fractions were pooled and dried *in vacuo,* reconstituted at 10 mg/ mL in 20-40% MeOH/ H_2O (BM-PF) and analysed by LC-FD under conditions suitable for PSTs as previously described (Chapter 4). Aliquots of BM-PF were then titrated against *B. marinus* STXBP extract (50 µg total protein) with 2 nM [^{3}H] STX in the MCE protein binding assay and sodium channel radio-receptor assays were conducted as previously reported (Chapter 2, 3, 4).

5.2.5 Lifecycle Analysis

The presence of STX binding activity over the lifecycle of *B. marinus* (see Fig. 5.2) was examined. Blood from five adults and 10 juvenile toads was collected by cardiac puncture and placed in lithium coated Vacutainer® tubes (BD). Plasma was obtained by centrifugation (2500 × g; 15 min.) at room temperature. Plasma was immediately pooled, filtered (0.22 µM) and frozen in $N_{2\,(l)}$ then freeze dried *in vacuo*. Several strings of *B. marinus* embryos were collected from the surface of stagnant water in Townsville during 2002 and maintained at AIMS during metamorphosis to confirm identification as described earlier (section 5.2.2). Sub-samples of these embryos (5 g) were extracted as previously reported (section 5.2.3). At least 15 toadlets (< 1 g each) and 15 toad-poles from three different

metamorphic stages (see Fig. 5.2), were pooled for protein extraction. All extracts and plasma were reconstituted at 10 mg (dry weight)/ mL in 20 mM HEPES, pH 7.4 containing cØmplete protease inhibitors and protein concentration determined by the method of (Bradford, 1976) with a BioRad Protein Assay (BioRad) and using bovine gamma globulin in the construction of the standard curve. All extracts were then titrated against [^{3}H] STX in the MCE protein binding assay, developed in Chapter 2.

5.2.5 Seasonal Variation

In order to examine the seasonal variation of STXBP yield in *B. marinus*, aliquots of plasma from all 40 adults collected over the course of this study were retained for radio-receptor assay. Toads were collected from a variety of sites within Townsville, QLD, as required during most months from between May 2002- April 2004. Plasma from each individual was titrated and assayed by MCE protein binding method as described earlier (section 5.2.3). Activity data was compared to Townsville daily (9 am) temperature records from the Australian Bureau of Meteorology observations for Townsville Aero station.

5.2.7 Protein Purification

Several chromatography purification strategies were trialled which included affinity (HiTrap Heparin; Amersham), ion exchange (HiTrap IEX Test Kit; Q, DEAE, SP and CM Sepharose; Amersham), hydrophobic interaction (HiTrap HIC Test Kit; Phenyl, Butyl and Octyl Sepharose; Amersham), chromatofocussing (PBE 118 with Polybuffer 96 and Pharmalyte pH 8- 10.5; Amersham), size exclusion (Sephacryl 300, Superdex 200HR; Amersham). From these extensive trials, a final 2-step purification strategy incorporating anion exchange on Q-Sepharose Fast Flow (Q-FF) media, followed by size exclusion chromatography on Superdex 200HR, was developed and optimised.

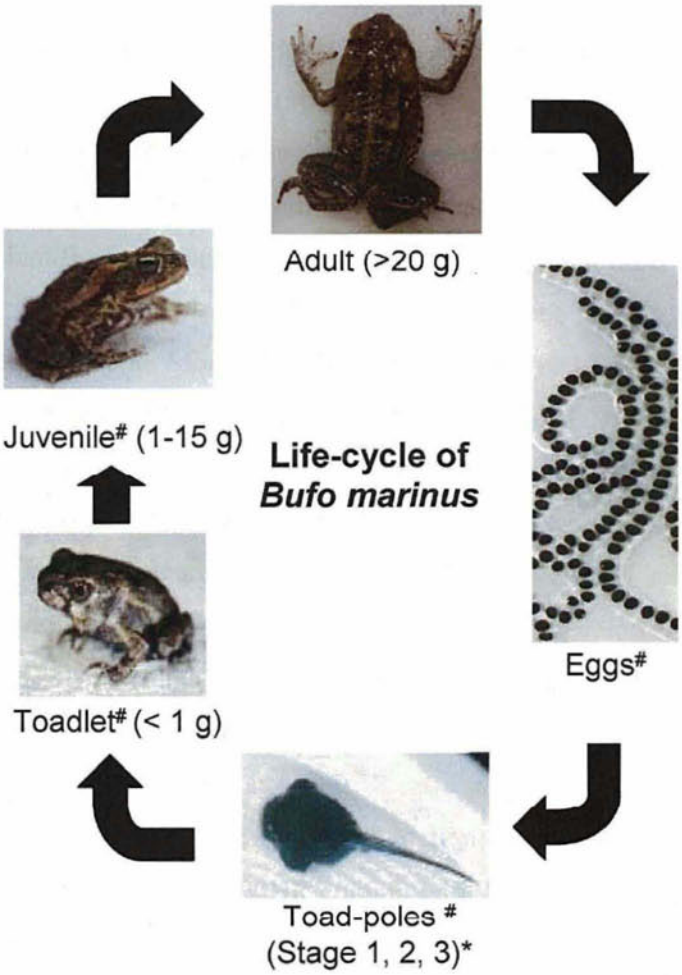

Figure 5.2 Lifecycle of the cane toad, *Bufo marinus*. *Stage 1 toad-poles are defined as the first emergent form; Stage 2 with hind legs; and stage 3 with hind and forelegs present. #Photographs obtained from Pergolotti, 2004 and used with permission.

Q-FF media (Q-FF; Amersham) was packed in a glass econo-column (BioRad) with final gel bed at 200 mm × 10 mm and equilibrated with 40 mL QS-buffer (20 mM piperazine-HCl, pH 9.8), then 40mL QE-buffer (20 mM piperazine-HCl; 1M NaCl, pH 9.8) followed by a final 40mL QS-buffer all applied at 0.5 mL/ min with a Shimadzu LC-10AT liquid chromatograph system, SCL-10AVp System Controller and using Shimadzu Class VP software (Version 6.12.SP2; Shimadzu Scientific Instruments, Oceania). Following column equilibration, freeze dried *B. marinus* plasma was reconstituted in QS-buffer (20 mM piperazine-HCl, pH 9.8; 50 mg/ mL), filtered (0.22 µM) and 500 µL applied to the top of the Q-FF column via a sample injection port. Protein separation was

achieved by 10% step gradient elution (20 mL per step) with QE buffer from 0-50% followed by a high salt wash (100% QE-buffer) followed by a final 20 mL wash with QS-buffer. All separations were carried out at a flow rate of 0.5 mL/ min. and protein peaks detected with a Shimadzu SPD-10A UV-Vis detector (Shimadzu Scientific Instruments, Oceania) at $UV_{280\ nm}$. Fractions (1 mL) were collected every 2 min. in deep well polyethylene plates with a Gilson 202C Fraction Collector (John Morris Scientific,Wiolloughby, NSW). STX binding activity was determined by MCE protein binding assay (see Chapter 2) following determination of protein concentration with Bradford reagent according the manufacturers instructions (BioRad). Filtrate buffer was exchanged into SE-buffer (50 mM sodium phosphate; 150 mM NaCl, pH 7) by size exclusion chromatography on a 5 mL HiTrap Sephadex G25 desalting column (25 mm × 16 mm; Amersham). The protein preparations were then concentrated, filtered (0.22 μM) and degassed under vacuum.

The second stage of *B. marinus* STXBP purification required pre-equilibration of a 300 mm × 10 mm Superdex 200HR column with 60 mL SE-buffer at 0.5 mL/ min. Filtered protein preparation (100 μL; 1 mg total protein) was loaded on to the column and eluted isocratically at a flow rate of 0.25 mL/ min with SE-buffer. Protein elution was monitored at $UV_{280\ nm}$ and fractions (1 mL) collected for protein determination and MCE protein binding assay. The chromatographic separation was calibrated using gel filtration molecular mass standards (Sigma) which included cytochrome c (12.4 kDa), carbonic anyhydrase (29 kDa), bovine serum albumin (BSA; 66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa) and blue dextran (2000 kDa). The gel filtration molecular mass standards were used to construct a standard linear regression curve by plotting the log of the known molecular mass of each protein against the retention time. Interpolation of the retention time corresponding to STXBP peak elution with the standard curve provided an estimate of STXBP size in kilodalton (kDa).

Protein separation and visualisation of purification progress was examined on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 10% (w/v) gels, by the method of Laemmli (1970) and silver stained based on the methodology of Heukeshoven and Dernick, (1985) with a Plus One Silver

Staining kit (Amersham Biosciences). The apparent molecular masses of proteins were estimated from a calibration curve obtained with the following marker proteins: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), fructose-6-phosphate kinase (84 kDa), BSA (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa) and glyceraldehyde-3-phosphate dehydrogenase (36 kDa).

5.2.8 Peptide digest and amino acid sequencing

Purified *B. marinus* STXBP was concentrated by freeze drying and reconstituted in 20% glycerol (w/v) then aliquots were then loaded into several lanes of a 10% SDS-PAGE gel for electrophoresis and stained with Coomassie Blue G250. Identical protein bands from multiple lanes, corresponding to the purified STXBP were excised with a sterile scalpel then pooled and sent to the Australian Proteome Analysis Facility (APAF; Macquarie University, North Ryde, NSW) for tryptic digestion and manual MS-MS peptide sequencing. The following details of peptide sequencing methodology were provided by Mr. Lewis Adler from APAF. Gel slices were de-stained then a 16 hour tryptic digest was conducted at 37 °C. The resulting peptides were extracted using H_2O:CH_3CN (9:1, + 0.1% formic acid) for 15 min. The digested peptides were separated by nano-LC using a CapLC system (Waters Corporation, Milford, MA, USA). Samples (2 - 5 μL) were injected onto a micro C18 pre-column for pre-concentration and desalted with 0.1% formic acid at 30 μL/ min. After a 3 min. wash the pre-column was switched into line with the analytical column containing C18 RP silica (Atlantis, 75 μm x 100 mm, Waters Corporation). Peptides were eluted from the column using a linear solvent gradient, with steps, from H_2O:CH_3CN (95:5; + 0.1% formic acid) to H_2O:CH_3CN (20:80, + 0.1% formic acid) at 200 nL/ min. over a 40 min. period. The LC eluent was subject to positive ion nanoflow electro-spray analysis on a Micro-mass QTOF Ultima mass spectrometer (Micromass, Manchester, UK). The QTOF was operated in a data dependant acquisition mode (DDA). In DDA mode a TOFMS survey scan was acquired (m/z 400-2000, 1.0 sec.), with the three largest multiply charged ions (counts >15) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 8 sec. (m/z 50-2000). The LC/MS/MS data was then manually sequenced using Biolynx software (Micromass, Manchester, UK).

5.2.9 Poly (A) RNA isolation from B. marinus *liver*

Poly (A^+) RNA was isolated from the tissue of *B. marinus* liver with a Micro Poly (A^+) Purist Kit (Ambion Inc). Freshly dissected liver from *B. marinus* was frozen in liquid nitrogen and ground with a pre-chilled mortar and pestle. Lysis Solution (600 µL, Ambion) was added to 50 mg of powdered liver and mixed thoroughly then diluted in 1.5 mL Dilution Solution (Ambion), mixed and centrifuged at 12 000 × g for 15 min. at 4 °C. Oligo (dT) cellulose was added to the cleared lysate and vortexed thoroughly then incubated for 15 min. at room temperature (~25 °C) with gentle agitation to facilitate maximal binding of poly (A^+) RNA to the oligo (dT) cellulose. Following centrifugation at 4000 × g for 3 min., supernatant was removed and 1mL Lysate Wash (Ambion) was added to the pellet, then vortexed and centrifuged for an additional 3 min. at 4000 × g. The pellet was then washed in 600 µL Lysate Wash (Ambion), vortexed and then Lysate Wash was removed by centrifugation at 5000 × g for 20 sec. at room temperature in a spin column. Poly (A^+) RNA was removed from the oligo (dT) cellulose following the addition of RNA storage solution (70 °C) and eluted from the spin column following centrifugation at 5000 × g for 20 sec. at room temperature which was repeated an additional two times. Final poly (A^+) RNA selection was performed according to the manufacturer's instructions (Ambion). RNA quantitation was performed by diluting an aliquot of the poly (A^+) RNA preparation in TE (10 mM Tris-HCl pH 8, 1 mM EDTA) and measuring the absorbance at $UV_{260\ nm}$ and $UV_{280\ nm}$ with a GeneQuant™ *pro* RNA/DNA Calculator (Amersham Biosciences). Purity was determined by assessment of the $UV_{260\ nm}$: $UV_{280\ nm}$ ratio.

5.2.10 First strand cDNA synthesis

First strand synthesis reactions were performed using 1 µg of poly (A^+) RNA (2 µL) from *B. marinus* liver, random hexamer primers (1 µL; Sigma-Genosys) and sterile water (2 µL). Components were mixed, centrifuged for 10 sec. at room temperature in a bench top micro-centrifuge (Eppendorf) then incubated at 70°C for 2 min. Samples were subsequently cooled on ice for 2 min. then centrifuged for a further 10 sec. The second step in the cDNA synthesis required the addition of 2 µL of 5 × BD First Stand Buffer (250 mM Tris-HCl, pH 8.3; 375 mM KCl; 30 mM $MgCl_2$), 1 µL dithiothreitol (DTT; 20 mM), 1 µL dNTP Mix (dATP,

dCTP, dGTP, and dTTP, each at 10 mM), and 1 µL BD PowerScript Reverse Transcriptase (BD) to a final reaction volume of 10 µL. Samples were air incubated at 42 °C for 90 min. then diluted with 250 µL Tricine-EDTA Buffer (10 mM Tricine-KOH (pH 8.5); 1.0 mM EDTA) and heated for 7 min. at 72 °C. Final cDNA stocks were then stored at -80 °C until use.

Verification of cDNA was performed by polymerase chain reaction (PCR) with eukaryote 18S forward and reverse primers kindly provided by Jennifer Swan from AIMS. The 20 µL reaction was performed in duplicate and contained H_2O (11.5 µL), Qiagen 5 × PCR buffer (2 µL), dNTP (1 µL), 10 µM of 18S forward and reverse oligonucleotides (1 µL of each) and cDNA (2.5 µL) then thermo-cycled (Table 5.1). Duplicate water controls were also performed as described with the omission of cDNA template. 5 µL aliquots of PCR products from these reactions were applied to a 1.5% TAE agarose gel then visualised and photographed under UV light.

Table 5.1 PCR cycling conditions used in verification of cDNA prepared from *B. marinus* liver with 18S forward and reverse primers.

PCR Step	Temp	Duration	No. cycles
Denaturation	94 °C	2 min.	1
Denaturation	94 °C	30 sec.	
Annealing	60 °C	30 sec.	30
Extension	72 °C	30 sec.	
Extension	72 °C	2 min.	1

5.2.11 Degenerate PCR

Four forward (AR_dF-01 to 04) and reverse degenerate primers (AR_dR-01 to 04) were designed (see Table 5.7 of Results) from back translated peptide sequences obtained from purified *B. marinus* STXBP digests (see Table 5.6 of Results) using the WebAngis back translation protocol (ANGIS, 2004). All back translations incorporating codon frequencies for *B. marinus* (NCBI, GenBank Release 139.0). All desalted oligonucleotides (see Table 5.7) were synthesised by Sigma-Genosys (Castle Hill, NSW). Preliminary 20 µL degenerate PCR reactions were performed using a QIAGEN PCR Core Kit (Clifton Hill, VIC) and Hot-Lid Themocycler (Eppendorf). Amplification trials consisted of non-redundant combinations of

forward and reverse primer pairs (0.5 μM each; Table 5.2) and reaction mixtures comprised 2 μL of 10 × PCR Buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$; pH 8.7; 20°C), 1 μL dNTP mixture (10 mM), 0.2 μL Taq DNA polymerase (5 U/ μL) and sterile deionised H_2O to a final volume of 20 μL.

Table 5.2 Degenerate primer combinations used in polymerase chain reaction of cDNA from the liver of *B. marinus*. Primers were designed from back translated peptide sequences from purified *B. marinus* STXBP digests and incorporated codon frequencies for *B. marinus*.

Combination ID	Degenerate primers	
	Forward	Reverse
1	AR_dF-01	AR_dR-02
2	AR_dF-01	AR_dR-03
3	AR_dF-01	AR_dR-04
4	AR_dF-02	AR_dR-01
5	AR_dF-02	AR_dR-03
6	AR_dF-02	AR_dR-04
7	AR_dF-03	AR_dR-01
8	AR_dF-03	AR_dR-02
9	AR_dF-03	AR_dR-04
10	AR_dF-04	AR_dR-01
11	AR_dF-04	AR_dR-02
12	AR_dF-04	AR_dR-03

PCR products (5 μL) were then applied to a 1.5% TAE Agarose gel containing 5 μM EtBr, buffer and run at 80 V for 30 min. Considerable optimisation of cycling conditions, Mg^{2+} and primer concentration was required to maximise PCR products. Successful PCR reactions contained 5 μL of each forward and reverse primer, 3.6 μL $MgCl_2$ (6 mM, final concentration) and cycled as detailed in Table 5.3. Final PCR products (20 μL) were electrophoresed on a 1.5% TAE agarose gel and all visible bands were excised under UV. Agarose was removed by centrifugation at 10,000 × g for 60 sec. through 3 mm Whatman paper and 1 μL of each gel purified PCR product was re-amplified in a 50 μL PCR optimised reaction using corresponding primer pairs and cycled as previously described (see Table 5.3). 1.5% TAE agarose electrophoresis was performed and all visible bands were excised and purified by centrifugation as described.

Table 5.3 Polymerase chain reaction thermo-cycling conditions used in preliminary and final experiments using degenerate primers designed from *B. marinus* STXBP peptide sequences and cDNA derived from *B. marinus* liver.

PCR Step	Preliminary Reaction		Optimised Reaction		No. cycles
	Temp	Duration	Temp	Duration	
Denaturation	94 °C	2 min.	94 °C	2 min.	1
Denaturation	94 °C	30 sec.	94 °C	30 sec.	30
Annealing	60 °C	30 sec.	48 °C	30 sec.	
Extension	72 °C	30 sec.	72 °C	2 min.	
Extension	72 °C	2 min.	72 °C	4 min.	1

5.2.13 Ligation and transformation reactions

Purified PCR products from degenerate PCR reactions were ligated using a pGEM®-T Easy Vector System II (Promega, Annandale, NSW) following the manufacturers protocol. Reactions (10 µL) containing 2 × Rapid Ligation Buffer (5 µL), pGEM®-T Easy vector (50 ng; 1 µL), T4 DNA Ligase (3 Weiss units/ µL; 1 µL) and purified PCR product (3 µL) optimised to a 1:1 vector:insert ratio. All components were mixed and incubated at 4 °C overnight then ligation reactions were centrifuged at room temperature for 60 sec. at 10,000 × g. Each reaction was then added to 50 µL JM109 high efficiency competent cells (Promega) and incubated for 20 min. on ice. Cells were subsequently heat-shocked for 45 sec. in a water bath at 42 °C, then immediately transferred to ice and incubated for 2 min. Filter sterilised SOC media (950 µL, room temperature; pH 7.0) containing Bacto®-tryptone (20 mg/ mL), Bacto®-yeast extract (5 mg/ mL), 10 mM NaCl, 2.5 mM KCl, 20 mM Mg^{2+} and 20 mM glucose, was then added to the ligation reaction transformations and shaken for 1.5 hrs. at 37 °C.

5.2.12 Cloning and Sequencing

Liquid Broth (LB) ampicillin plates (LB/ampicillin) were prepared by adding 15 g agar to 1 L of LB medium (10g/ L Bacto®- tryptone; 5g/ L Bacto®- yeast extract ; 5g/ L NaCl) which was autoclaved, then allowed to cool to 50 °C prior to the addition of ampicillin (100 µg/ mL). Approx. 30 mL of LB/ampicillin media was then poured into 85 mm plates and allowed to harden. Prior to the culture of transformed cells, plates were supplemented with 100 mM Isopropyl-beta-D-

thiogalactoside (IPTG; 100 µL) and 50 mg/ mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 20 µL) and incubated at 37 °C for 30 min. 100 µL aliquots of transformation cultures were then plated onto duplicate LB/ampicillin/IPGT/X-Gal plates and incubated overnight at 37 °C. Three plaques from white colonies were randomly picked from each plate (6 plaques per transformation) and introduced to 30 µL sterile H_2O by gentle vortex. An additional two white and two blue colonies from control DNA ligation reaction transformation plates were also selected to act as positive and negative controls in subsequent PCR reactions. PCR amplification of cloned inserts was performed using prepared plasmid as DNA template (2 µL), 10 mM USP and RSP oligonucleotide primers (1 µL of each), Qiagen 10 × PCR buffer (2 µL), 10 mM dNTPs (1 µL), *Taq* DNA polymerase (0.2 µL) and sterile H_2O (12.8 µL) then cycled with a Eppendorf Hot-Lid PCR System thermocycler (Table 5.4). DNA gel electrophoresis of plasmid PCR products was performed using a 1.5 % TAE agarose gel containing 0.5 µg/ mL ethidium bromide. 52 bands, corresponding to their expected insert size, were excised from the gel under UV light and products eluted from the agarose by centrifugation through 3 mm Whatman filter paper. Clarified products were then purified further using a QIAquick® PCR purification kit (QIAGEN, Clifton Hill, VIC) according to the manufacturer's instructions and quantitated by UV absorbance at $UV_{260\ nm}$ and $UV_{280\ nm}$ with a GeneQuant™ *pro* spectrophotometer.

Table 5.4 Thermo-cycle conditions employed for PCR amplification of cloned inserts from JM109 high efficiency competent cells with USP and RSP primers.

PCR Step	Temp	Duration	No. cycles
Denaturation	94 °C	5 min.	1
Denaturation	94 °C	30 sec.	
Annealing	55 °C	30 sec.	30
Extension	72 °C	2 min.	
Extension	72 °C	2 min.	1

Sequencing reactions of purified PCR products containing plasmid inserts were prepared with a DYEnamic ET dye terminator cycle sequencing kit (DYEnamic ET; Amersham Biosciences) using USP primer and purified PCR products from plasmid as template. The final nucleotide sequencing reaction volume was 10 µL

for inserts less than 500 bp (4 µL DYEnamic ET sequencing reagent pre-mix), 15 µL reactions where inserts between 500-1000 bp (6 µL DYEnamic ET), and 20 µL for inserts larger than 1000 bp (8 µL DYEnamic ET). In all cases reactions were processed in an Eppendorf Hot-Lid PCR thermocycler programmed for 30 cycles at 95 °C for 20 sec., 50 °C for 15 sec. and 60 °C for 1 min. Subsequently, products were purified by centrifugal elution and washed through a 0.7 mL slurry of Sephadex G50 fine grade (3 g/ 45 mL H_2O; Amersham) to a final volume of 30 µL. Purified reaction products were analysed with an Amersham MegaBACE 1000 DNA analysis system by Dr. Lynn Woodward at the Genetic Analysis Facility (GAF) within the Advanced Analytical Centre (AAC) at James Cook University (JCU), Townsville, Queensland, Australia. The resulting chromatograms were compared, trimmed, and analysed using Sequencher™ software (Genecodes Corporation, Ann Arbor, MI, USA).

5.2.13 Bioinformatics and data analysis

Statistical analyses such as paired and unpaired t-test, ANOVA and correlation were performed using GraphPad InStat® version 3.05 for Windows (GraphPad software, San Diego California USA; www.graphpad.com). Radio-receptor assay data was analysed by non-linear regression integrated with one-site competition models and linear regression of protein standard curves was performed using GraphPad Prism® version 3.00 for Windows (GraphPad software, San Diego California USA, www.graphpad.com). Experimental nucleotide sequences were scrutinised in Sequencher™ (Version 4.14; GeneCodes Corporation, Ann Arbor, MI, USA) and removal of vector contamination, trimming and editing by contiguous sequence (Contig) analysis was performed. Homologies of the deduced amino acid sequences were determined using BLASTX and BLASTP analysis (Altschul et al., 1997) in BioManager (Version 2.0) by the Australian National Genomic Information Service (ANGIS). Multiple sequence alignments were produced using CLUSTALW (Thompson et al., 1994) and reported using BoxShade (Hofmann and Baron, 2004). Identification and analysis of open reading frames of translated protein sequences was performed using FLIP protocols (Brossard, 1997) in BioManager (ANGIS, 2004).

5.3 Results

5.3.1 Tissue Distribution of STX Binding Activity in Bufo marinus

The distribution of STX binding activity in *Bufo marinus* tissues was investigated with the aid of the MCE protein binding assay developed and optimised in Chapter 2. STX binding activity was detected in all extracted tissues with the exception of the venom gland (see Table 5.5) and the highest yields were identified in non-excitable tissue including plasma, kidney, unfertilised eggs, lung and heart. Specific activity ranged from 0.9 ± 0.05 pmol/ mg in extracts of skeletal muscle to 6.1 ± 0.2 pmol/ mg in lung extracts.

5.3.2 Distribution and Isolation of a STXBP ligand from B. marinus

Inhibition of [^{3}H] STX and STXBP extract binding was observed in MeOH extracts of *B. marinus* in a preliminary investigation (see Chapter 3) and was examined further during this study. MeOH extracts from a variety of tissues from five adult cane toads were prepared and assayed in the MCE protein binding assay. Inhibition of [^{3}H] STX binding (5 - 30 % mean) was confirmed in MeOH extracts derived from venom, heart, intestine, lung, kidney, liver and plasma (see Fig. 5.3A). Based on this activity, viscera extracts were prepared from an additional 5 animals and fractionated by C18 SPE chromatography.

Inhibitory STX binding activity was identified at 20-40% MeOH (see Fig. 5.3b) which was then pooled and concentrated for further assay and LC-FD analysis. No PST or TTX related peaks were identified by LC-FD analysis. Likewise, no PST or TTX like activity was observed in the sodium channel assay. MCE assay titration of the pooled active fraction revealed competitive binding that was fitted to a non-linear regression one-site model of competition (see Fig. 5.4) but did not reach 100% inhibition as compared to unlabeled STX control. The IC_{50} value of 74 ± 2 µg/ mL was calculated by curve fitting from the non-linear regression (see Fig. 5.4).

Table 5.5 Tissue distribution of saxitoxin binding activity in *Bufo marinus*.

Tissue/ organ	Yield (pmol/ g tissue)	Specific Activity (pmol/ mg protein)
Plasma	432 ± 2*	14 ± 1
Liver	100 ± 7	1.1 ± 0.1
Kidney	167 ± 3	5 ± 0.2
Lung	155 ± 5	6.1 ± 0.2
Stomach	67 ± 3	1.9 ± 0.1
Intestine	80 ± 2	2.5 ± 0.1
Testes	90 ± 2	3.9 ± 0.1
Eggs	159 ± 3	4.9 ± 0.2
Venom gland	-	-
Brain	17 ± 2	1.1 ± 0.1
Heart	126 ± 4	5.5 ± 0.1
Skeletal muscle	25 ± 3	0.9 ± 0.05
Skin	23 ± 3	1.2 ± 0.05
Tongue	43 ± 2	1.4 ± 0.1

- denotes that activity was absent or below detection limits
* calculated as pmol/ mL plasma

5.3.3 Lifecycle and seasonal variation of STX binding activity in B.marinus

STX binding activity was present at all metamorphic life stages of *B. marinus* with little variation in the yield and specific activity of each extracted group (Fig. 5.5). Aliquots of plasma were collected from all *B. marinus* individuals used during the course of this study (n=40). In all cases, plasma was isolated from whole blood on the same day of collection and the dates recorded from each individual were compared to daily temperature records for Townsville to examine any seasonal variation in the presence or activity of STXBPs from *B. marinus*. Daily temperatures were converted into monthly averages which correlated to reduced STX binding activity in June and July of 2002 and 2003 that was significant ($p<0.05$; ANOVA; Fig. 5.6A). Translation of monthly temperature averages to corresponding season also demonstrated that STX binding activity was reduced in *B. marinus* specimens collected in winter (see Fig. 5.6B).

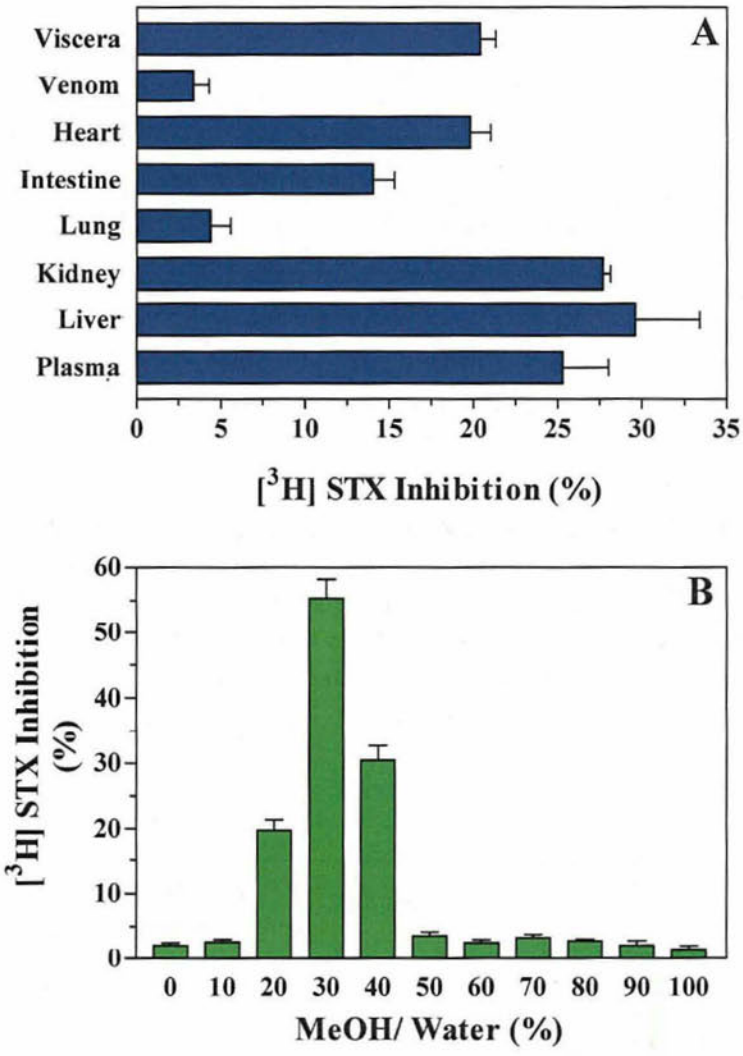

Figure 5.3 Inhibitory STX binding activity detected in MeOH extracts of *B. marinus* tissues (A) and following fractionation of viscera extract on C18 (B) in the presence of *B. marinus* STXBP extract. Data are represented as the mean ± SEM of two experiments performed in duplicate. MeOH fractions in B, were from MeOH, 10% step gradient elution on C18 cartridges by solid phase extraction chromatography.

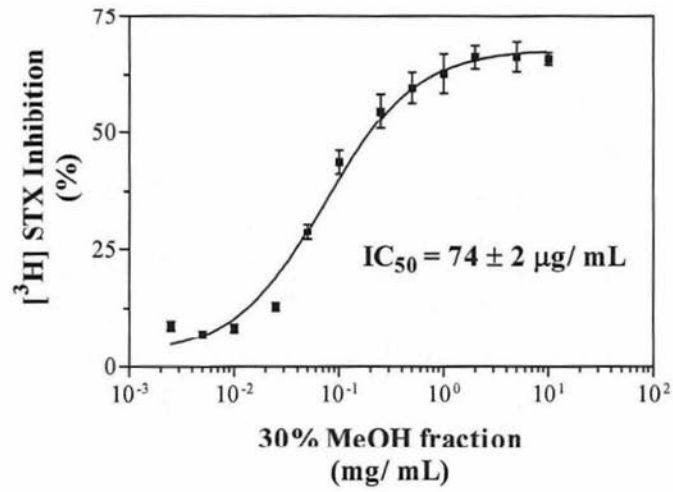

Figure 5.4 Titration of 30% methanol fraction obtained following C18 SPE chromatography of *B. marinus* MeOH viscera extract in the presence of *B. marinus* STXBP extract. Data are shown as the mean ± SEM of two MCE protein binding assays performed in duplicate with 2nM [^{3}H] STX .

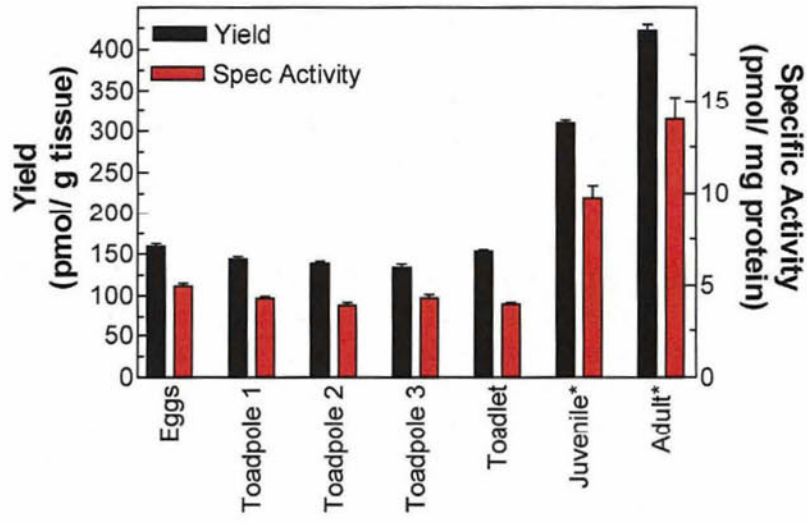

Figure 5.5 Levels of saxitoxin binding activity observed at various stages of metamorphosis in the cane toad, *Bufo marinus*. Data are mean ± SEM from three MCE protein binding assays performed in duplicate. * denotes that yield was calculated as pmol/ mL plasma.

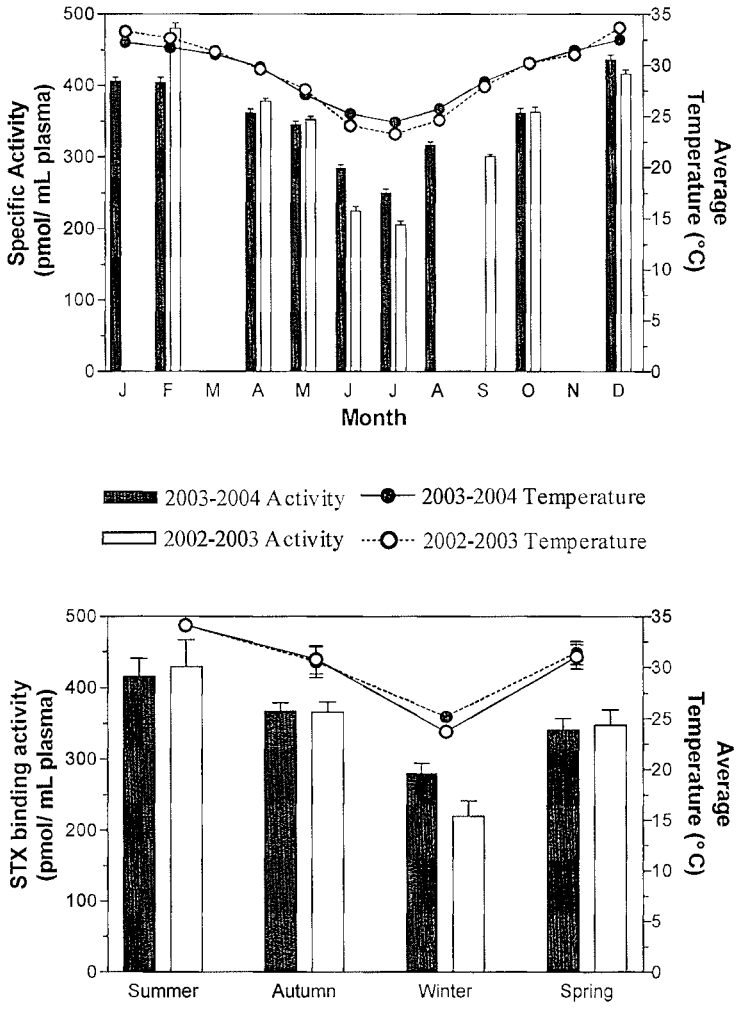

Figure 5.6 Monthly (A) and seasonal (B) variation in saxitoxin binding activity from the plasma of the cane toad *Bufo marinus* with daily environmental temperature from Townsville between 2002 to 2004. Plasma from a total of 40 adult toads was assessed over the course of the study. Activity is represented as the mean ± SEM from three MCE protein binding experiments performed in duplicate. Temperature data were obtained from the Australian Bureau of Meteorology 9 am daily observations for Townsville Aero station. Data from summer (Dec-Feb), autumn (March-May), winter (June-August) and spring (September–November) were collated for seasonal adjustments.

5.3.4 Purification of a STXBP from B. marinus plasma

Following extensive purification trials, *B. marinus* STXBP was purified by a two-step purification scheme incorporating anion exchange followed by high resolution size exclusion chromatography (SEC). Protein separation from *B. marinus* plasma on the strong anion exchanger, Q-FF, resulted in early elution of STX binding activity at 30 % NaCl which corresponded to a protein peak with a retention time of 65 min. (see Fig. 5.7). Active fractions were then pooled, desalted and concentrated prior to SEC on a Superdex 200HR column (see Fig. 5.8A).

STX binding activity was detected in five fractions following protein elution on Superdex 200HR and corresponded to a protein peak with a retention time of 67 min. and indicated a molecular weight of 87 ± 5 kDa when compared to the Superdex 200HR protein standard curve (see Fig. 5.8B). Proteins in the final pooled active fractions were separated by 10 % SDS-PAGE and silver stained resulting in a single band with an estimated size of 93 ± 3 kDa when compared to the standard curve (see Fig. 5.9).

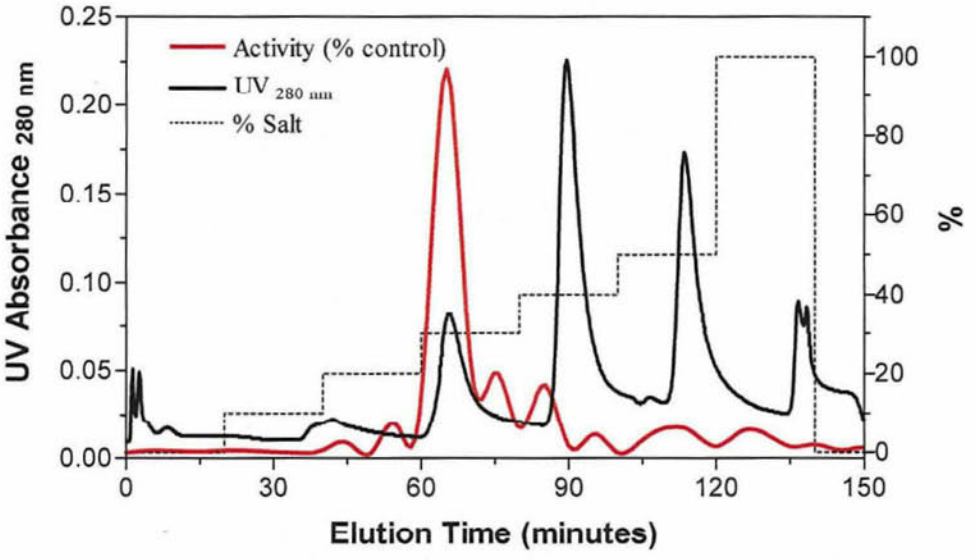

Figure 5.7 Protein separation of crude plasma from Bufo marinus by anion exchange chromatography on a column of Q-Sepharose Fast Flow. Protein separation was achieved at 0.5 mL/ min with 20mM Piperazine pH 9.8 and eluted with a NaCl gradient (0-50%) with protein peaks monitored at $UV_{280\ nm}$. Saxitoxin binding activity in 1 mL fractions was determined with the MCE protein binding assay.

Additional 10% SDS-PAGE was performed using pooled and concentrated aliquots of purified *B. marinus* STXBP. Following separation identical bands corresponding to the purified STXBP from several lanes were stained with Coomassie Blue G250 then excised, pooled and sent to APAF for tryptic digestion and MS-MS amino acid sequencing resulting in 3 verified peptide sequences (Table 5.6).

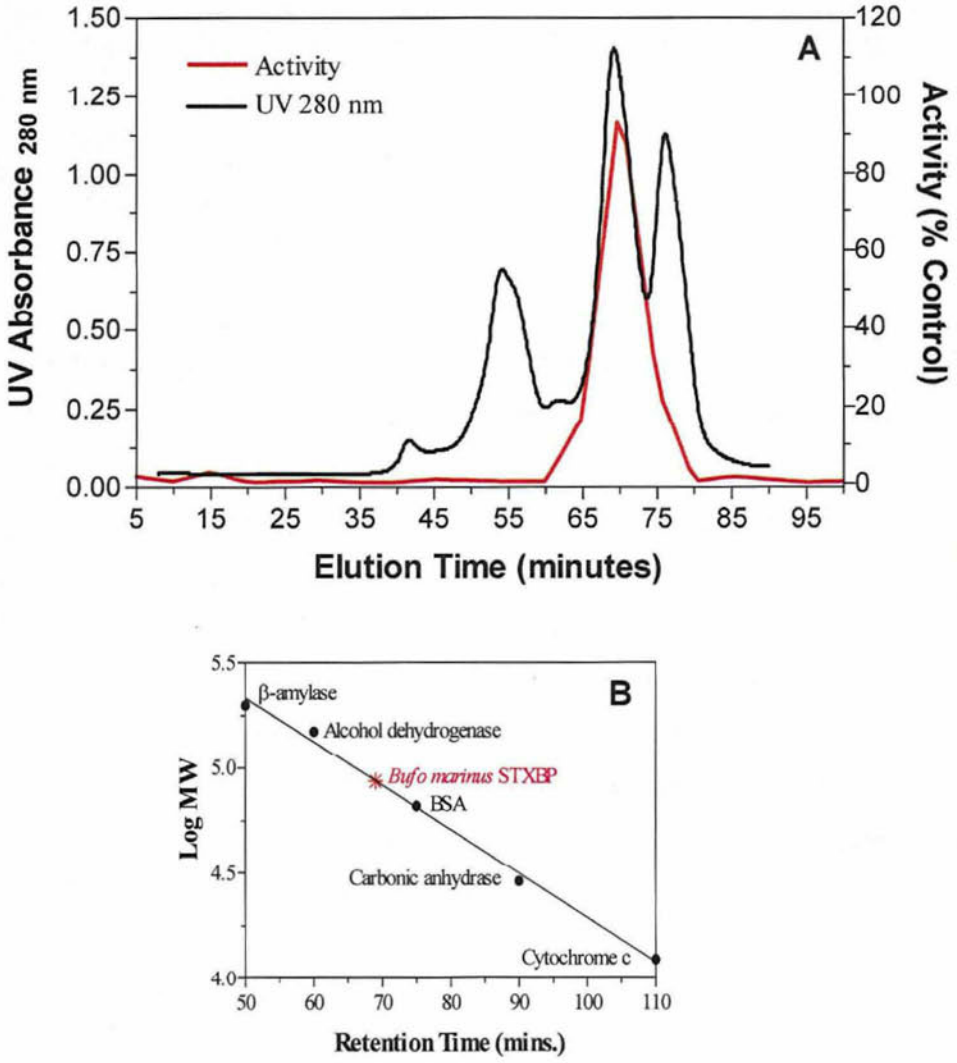

Figure 5.8 Purification (A) and size estimation (B) of STXBP from *B. marinus* plasma by Superdex 200HR size exclusion chromatography. Pooled, desalted, active fractions from Q-Sepharose column were loaded onto the Superdex column and eluted isocratically with 50mM sodium phosphate, 150mM NaCl, pH 7.0 at 0.25 mL/ min. Protein elution was monitored by UV_{280} and activity measured using the MCE protein binding assay. Size estimation was determined by interpolation of active peak retention time from linear regression of MW standard protein elution.

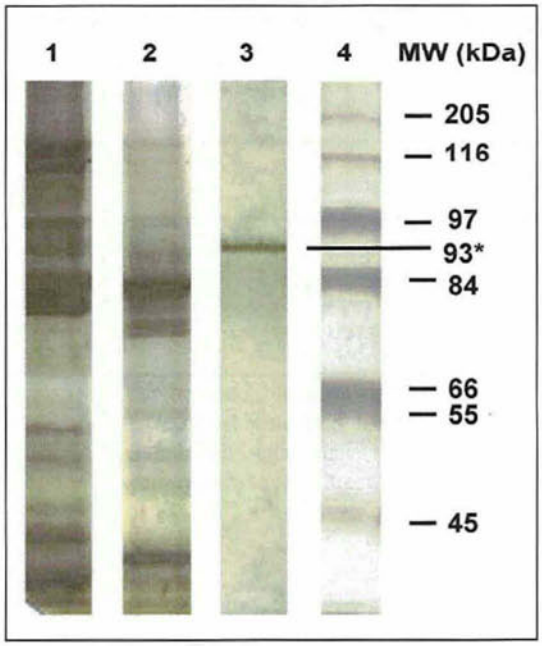

Figure 5.9 Silver stained 10% SDS–PAGE of STXBP purification from *B. marinus* plasma, performed under denaturing conditions. Lane 1, Protein elution from crude plasma; Lane 2, Q-Sepharose pooled active fractions; Lane 3, Superdex 200HR pooled active fractions; Lane 4, molecular weight standards. STXBP size estimation was calculated from standard curve as described in "Methods".

5.3.5 Partial Sequencing of a STXBP from B. marinus

Poly (A^+) RNA was isolated from *B.marinus* liver tissue and then reverse translated to obtain first stand cDNA (see Fig. 5.10) which was then used as a template for degenerate PCR. Four forward and four reverse degenerate primers were designed based on the back translated peptide sequences obtained from APAF (see Table 5.6) incorporating *B. marinus* codon frequencies (see Table 5.7). Considerable optimisation of PCR conditions was required to allow amplification from cDNA with degenerate primer pairs. Maximal yields of PCR products were recovered by increasing the Mg^{2+} concentration in the PCR reaction from 1.5 mM to 6 mM (see Fig. 5.11). All visible bands were then excised from the agarose gel under UV light and purified products were re-amplified by PCR (Fig. 5.12).

Table 5.6 Peptide sequences resulting from MS-MS analysis of purified B. marinus STXBP tryptic digests. Amino acid sequencing was performed and supplied by APAF.

Peptide ID	Sequence	Precursor m/z	Calculated MW
AR_P-01	VANMND[F/Mo][L/I]GK	554.76	1107.51
AR_P-02	{EV}VVN[L/I][L/I]NEQ{Q[L/I]}K	763.44	1524.87
AR_P-03	TGSYYAV[L/I]AVK	800.93	1599.84

[L/I] = Leucine and Isoleucine cannot be distinguished by MS
[F/Mo] = Phenylalanine and Methionine sulphoxide differ by 0.04 Da and could not be distinguished by MS analysis.
{ } = low confidence assignments

Table 5.7 Degenerate forward and reverse oligonucleotides designed from back translated peptide sequences from purified *B. marinus* STXBP incorporating codon frequencies for *B. marinus*.

Primer ID	Sequence (5'-3')
AR_dF-01	GTIGCIAAYATGAAYGAYWTBHT
AR_dF-02	GTIGTIAAYYTIYTIAAYGARCA
AR_dF-03	ACIGGIWSITAYTAYGCIGTIHTNGC
AR_dF-04	TAYTAYGCIGTIHTIGCIGTNAA
AR_dR-01	ADVAWRTCRTTCATRTTIGCNAC
AR_dR-02	TGYTCRTTIARIARRTTIACNAC
AR_dR-03	GCIADIACIGCRTARTAISWICCNGT
AR_dR-04	TTNACIGCIADIACIGCRTARTA

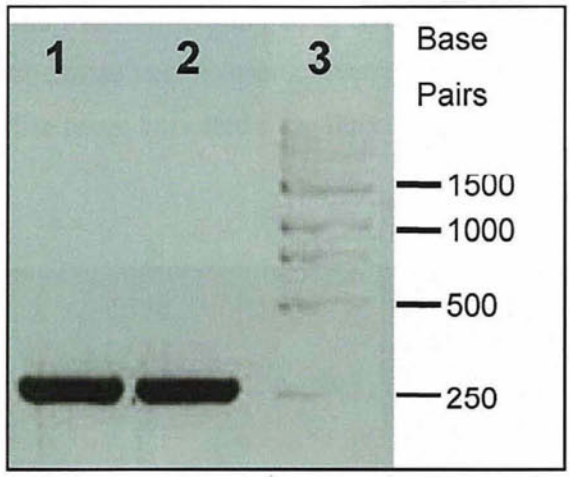

Figure 5.10 First strand cDNA synthesis from *B. marinus* liver (Lane 1) compared to control cDNA from *P. monodon* (Lane 2) and DNA ladder (Lane 3).

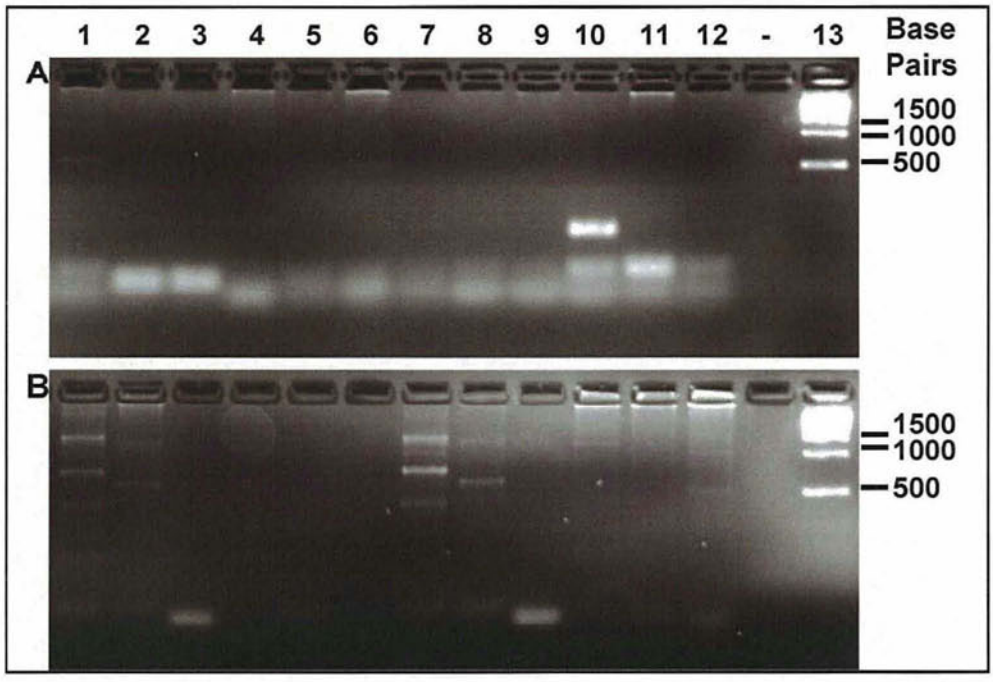

Figure 5.11 PCR amplification of cDNA from *B. marinus* liver with degenerate primer combinations in the presence of 5.5 mM Mg^{2+} (A) and 6 mM Mg^{2+} (B). Primer combinations 1-12 (see Table 5.2) were loaded into corresponding lanes and amplified fragments compared to 1kB DNA ladder (Lane 13). PCR cycling conditions were optimised as described in Table 5.3 of "Methods".

Eleven distinct bands resulted and were excised from the gel and purified, ligated and transformed into competent cells (*E. coli*; JM109) for cloning. Amplification of cloned vector inserts revealed more than 50 PCR products that were within the size range expected from the corresponding ligations (see Fig. 5.13 for examples).

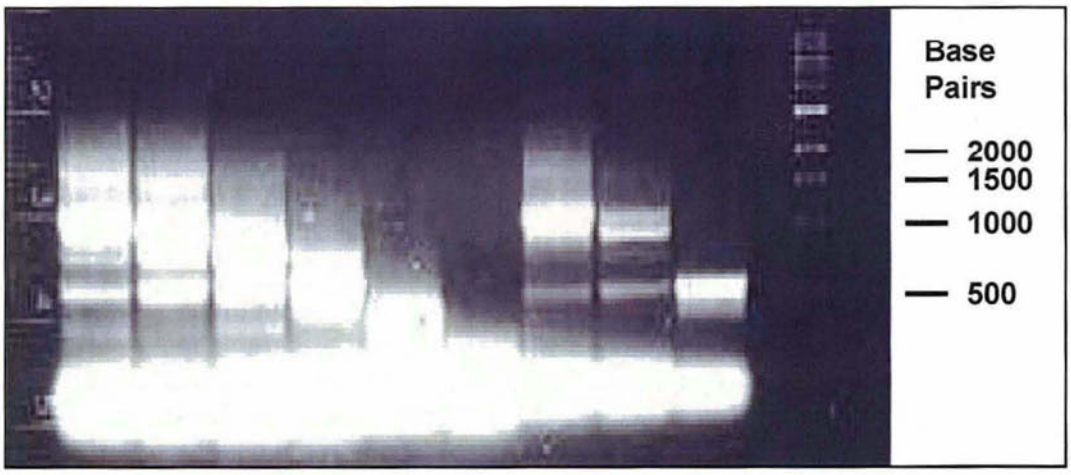

Figure 5.12 Re-amplified PCR products from degenerate PCR of cDNA from *B. marinus* liver in the presence of 6mM Mg^{2+}.

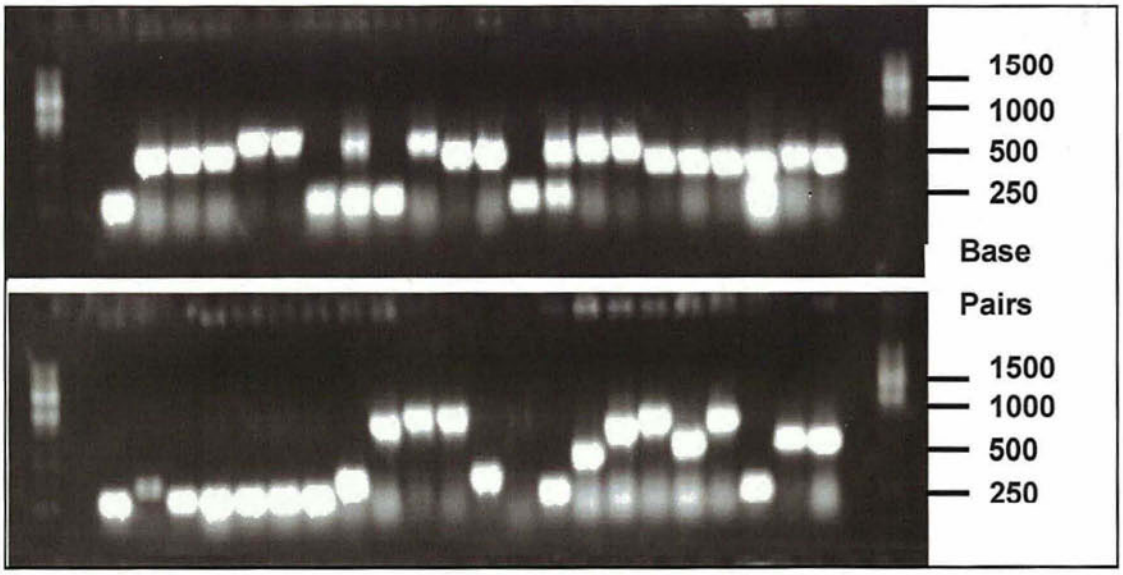

Figure 5.13 Representative PGemT® Easy cloned inserts from degenerate PCR amplification of cDNA fro *B. marinus* liver, amplified with USP and RSP primers.

Primary analysis of the nucleotide sequences of these products included removal of vector contamination, spectral editing and trimming of the 3' and 5' ends which resulted in 37 fragment sequences. BLASTX analysis revealed that 14 sequences were homologous to transferrin (TF) and SXPN and a further seven that were unrelated to known proteins. Almost 20% of the best hits from the database searches were from proteins isolated from amphibians including *Xenopus laevis* and *Rana catesbeiana* (see Appendix 4). Secondary Contig analysis (Sequencher) of the cloned fragments resulted in 15 partial sequences with the best hits from BLASTX results revealing sequence homology to TFs in six instances (see Table 5.8).

Table 5.8 Summary of BLASTX results of final nucleotide sequences resulting from cloning of degenerate PCR fragments into pGEM®-T Easy vector. Vector contamination was removed, 3' and 5' ends trimmed and sequences edited by Contig analysis in Sequencher® prior to BLASTX analysis as described in 'Methods'.

ID	Bases *	SwissProt Accession ID	Best Hit Description†	Best Hit Species‡	E Value§
AR_S01	639	Q9PT55	transferrin	*Oncorhynchus tschawytscha*	3^{e-22}
AR_S02	164	Q6IP81	mgc78867 protein	*Xenopus laevis*	1^{e-40}
AR_S03	550	Q6BCV7	rim2(+44a)	*Mus musculus*	4.5
AR_S04	1587	Q8RLS6	Nitrite reductase	*Pseudomonas mendocina.*	1
AR_S05	1110	TRFE_XENLA	Serotransferrin	*Xenopus laevis*	2^{e-26}
AR_S06	677	Q7TSX8	Transferrin	*Marmota monax*	2^{e-4}
AR_S07	1115	Q9PU66	Transferrin	*Salmo trutta*	2^{e-19}
AR_S08	1205	Q7T193	Transferrin	*Pagrus major*	5^{e-28}
AR_S09	1170	P79954	bmp type II receptor	*Xenopus laevis*	3^{e-24}
AR_S10	688	Q7ZYF4	gfpt1-prov protein	*Xenopus laevis*	2^{e-15}
AR_S11	623	VTDB_HUM	Vitamin d-binding protein	*Homo sapiens*	1^{e-08}
AR_S12	628	Q9W6F5	Vitamin-d binding protein	*Gallus gallus*	4^{e-12}
AR_S13	1210	n/a	No Hits	n/a	100.0
AR_S14	1419	Q6A169	Iron binding protein	*Chrysemys scripta elegans*	3^{e-25}
AR_S15	1305	Q9PLM4	Hypothetical protein	*Chlamydia muridarum*	2.2

* Number of nucleotides
† Descriptions are truncated from BLASTX results performed in BioManager by ANGIS.
‡ Species from which best hit record was obtained
§ Expectation value which represents the statistical significance threshold for reporting matches against database sequences.
n/a not applicable

Of these 15 sequences only one was not related to known proteins from Swiss-PROT or Sp-TrEMBL databases. A nucleotide sequence alignment (CLUSTALW) of these partial sequences and bullfrog SXPN confirmed similarities between fragments to the bullfrog SXPN nucleotide sequence (see Fig. 5.14). Nucleotide sequences deemed homologous to the TF family proteins were translated in 6 frames (-3 to +3) using Flip-6-Frames (Brossard, 1997) to determine the most likely open reading frames in each case and were confirmed by BLASTP analysis. Subsequent sequence alignment of deduced amino acid sequences with bullfrog SXPN (RC-SX) and a variety of TFs including *Xenopus laevis* serotransferrin (XL-ST), *Gallus gallus* ovotransferrin (GG-OT; chicken) and human serum TF (HS-ST), lactotransferrin (HS-LT) and melanotransferrin (HS-MT), revealed highest sequence identity in a region spanning approximately 150 amino acids.

The amino acid sequence derived from AR_S08 showed the highest homology with up to 55% sequence identity to both *R. catesbeiana* SXPN and *X. laevis* sero-transferrin. Several amino acid residues of AR_S08 aligned with highly conserved residues reported to be involved in Fe_3^+/ HCO_3^- binding in the putative C-lobe of TFs (Anderson et al., 1989; Baker et al., 1996); see Fig. 5.15)). Several cysteine residues of AR_S08 also aligned at positions conserved in members of the TF family that were examined and bullfrog SXPN (see Fig. 5.15).

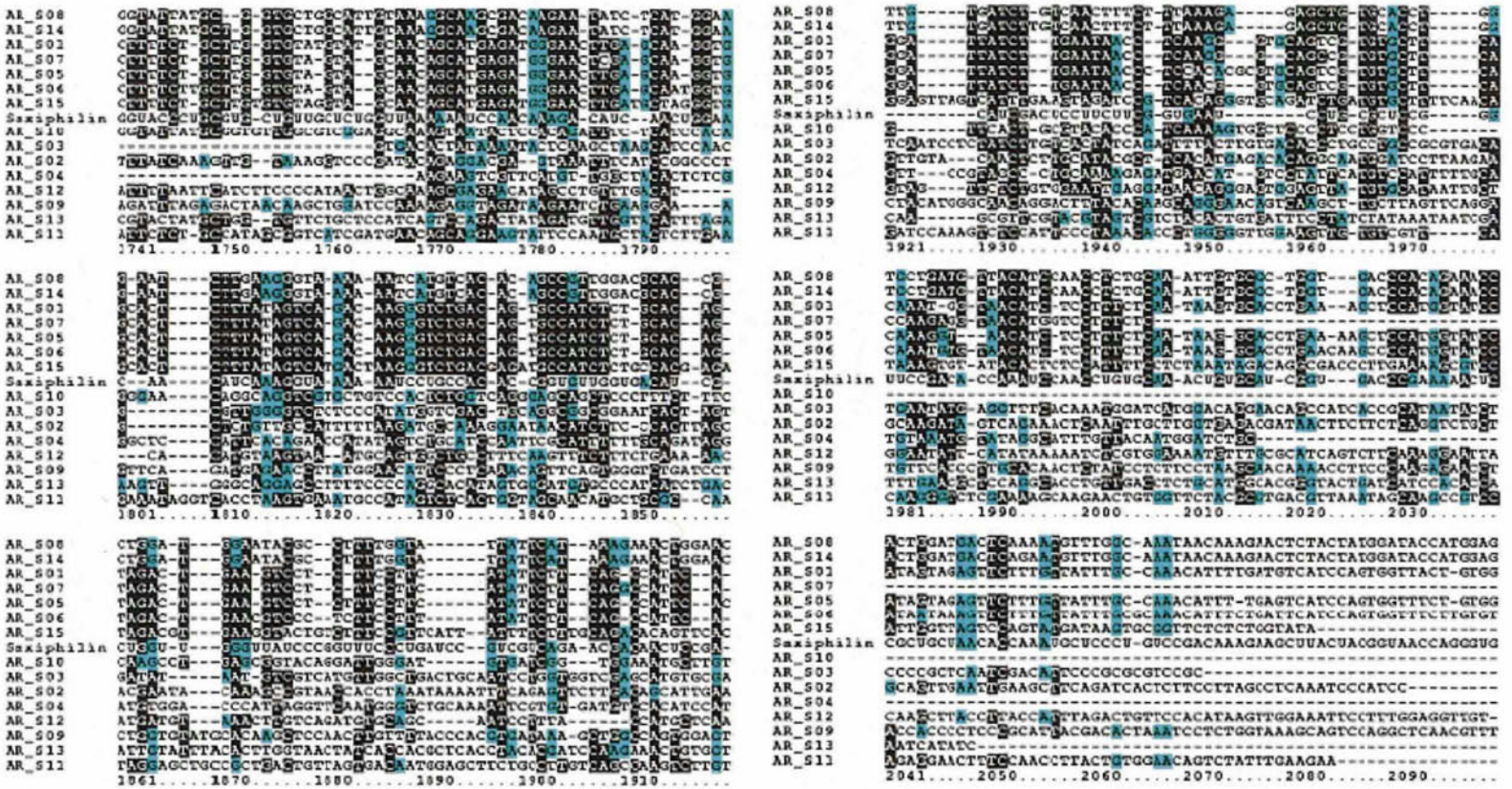

Figure 5.14 Multiple nucleotide sequence alignment of partial STXBP sequences from *Bufo marinus* compared to saxiphilin from *Rana catesbeiana*. Prior to alignment by Clustal W in BioManager (ANGIS), vector contamination was removed from experimental sequences which were then edited by Contig analysis in Sequencher® (GeneCodes) to produce 15 partial sequences (AR_S01-15) for alignment. BoxShade was used for alignment visualisation. For presentation purposes, only the most homologous regions of the alignment showing identical (black) and similar (cyan) sequence identities are illustrated.

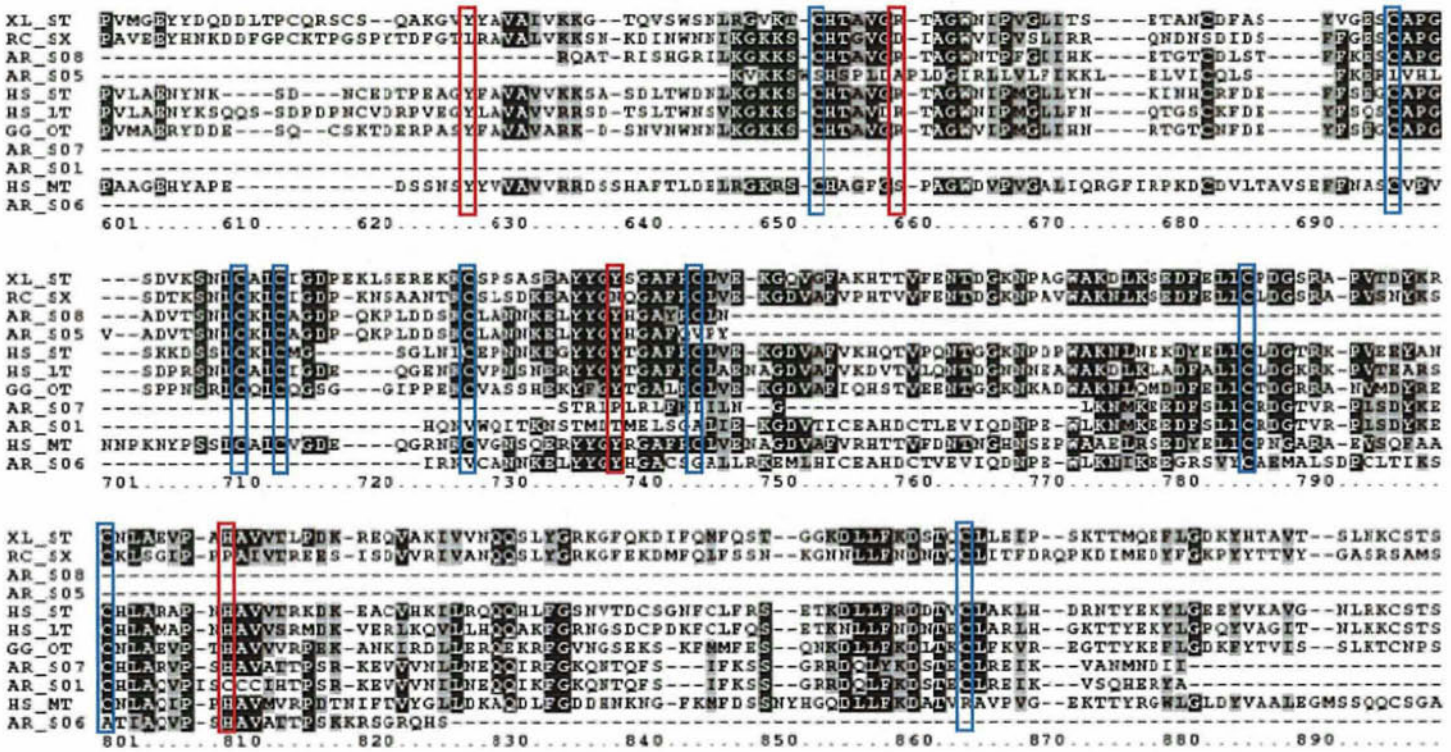

Figure 5.15 Protein sequence alignment of *R. catesbeiana* saxiphilin (RC-SX), *X. laevis* serotransferrin (XL-ST; frog), *G. gallus* ovotransferrin (GG-OT; chicken) and human serum transferrin (HS-ST), lactotransferrin (HS-LT), melanotransferrin (HS-MT) and selected experimentally determined amino acid sequences derived from *B. marinus* (AR_S01, AR_S05, AR_S06, AR_S07, AR_S08). Translated open reading frames of experimental nucleotide sequences deemed most homologous to transferrins are shown and were obtained using the Flip-6-frames protocol in BioManager by ANGIS. The gapped sequence alignment was performed with CLUSTALW and reported with BoxShade using BioManager (ANGIS). For presentation purposes, the most homologous region of the alignment, corresponding to the putative c-lobe of transferrins and saxiphilin is illustrated, with identical (black) and similar (grey) amino acid residues highlighted. Highly conserved residues involved in Fe_3^+/ HCO_3^- binding in transferrins are highlighted in red and conserved cysteine residues of several disulphide bonds of the c-lobe of transferrins are highlighted in blue.

5.4 Discussion

5.4.1 Tissue and Lifecycle Distribution of STX Binding Activity

Tissue distribution of STX binding activity in *Bufo marinus* was highest in non-excitable tissues such as plasma, kidney, lung, oocytes, liver and heart (see Table 5.5), most of which are notably disparate to the distribution of voltage gated sodium channels (VGSC) which in vertebrates are predominantly located in nerve, heart and skeletal muscle (Goldin, 1999). Similar findings were reported for SXPN distribution in *Rana catesbeiana,* where STX binding activity was present in all tissues that were examined and highest levels recorded for plasma, kidney, lung, heart and eggs (Mahar et al., 1991). The presence of plasma in heart, and particularly in highly vascularised tissue such as the lung, may have contributed to the observed increase in STX binding levels from these tissues. The only tissue extract identified without STX binding activity in this study was from the toad venom gland and this was most likely due to the incompatibility of a hydrophilic protein in a hydrophobic environment such as the lipid filled parotid glands. These studies do not reveal the location of STXBP production in *B. marinus* but the appearance of the protein in such a wide variety of tissues suggests that a relationship to the ubiquitous mobile TFs including bullfrog SXPN is more likely than a relationship to the VGSC.

Bufo marinus oocytes, embryos, toadpoles at three distinct metamorphic stages, toadlets, juveniles and adults all demonstrated STX binding activity within the same order of magnitude (see Fig. 5.5) indicating that the protein is required throughout life in these animals. It is interesting that STX binding activity was present in the oocytes, which are metabolically inactive, and may suggest a different biological role of the protein in this species. This investigation of STX binding activity over all life stages of *B. marinus* was the first thorough analysis of this kind that has been reported for any species identified with STX-specific activity to date. One other study also reported the presence of STX binding activity in adults and tadpoles of *R. catesbeiana* but the metamorphic stage of the tadpoles was not described and examination of froglet and juvenile frogs was never examined (Mahar et al., 1991).

5.4.2 Seasonal variation in STXBPs from B. marinus *plasma*

Bufo marinus STXBPs from the plasma of 40 adult toads collected over the course of this study revealed that animals collected in winter months such as June and July had consistently lower yields of STX binding activity than in summer months. The dates of collection and extraction of plasma from these animals was compared to daily temperature records for Townsville (9 am) where all animals were obtained. This data confirmed the correlation between low environmental temperature and apparent down–regulation of STX binding activity and in June-August of 2002 and 2003, this association was statistically significant ($p<0.05$; ANOVA). This result begs the question; does this statistical significance have any genuine relevance to the biological role of STXBPs in toads? Clearly, it is a possibility which merits further investigation, particularly as all animals identified with STX binding activity have been ectothermic and the absence of STX binding activity in warm blooded animals has been noted by other researchers (Llewellyn et al., 1997). Direct studies of this phenomenon should be carried out with greatly increased numbers of individuals under climate controlled conditions to assess whether this is, in fact, a valid hypothesis.

5.4.3 Distribution and Isolation of a STXBP ligand from B. marinus

Inhibitory STX activity was first recognised in MeOH extracts of *B. marinus* viscera in Chapter 3. Further examination of this activity in pooled MeOH tissue extracts from the toad revealed the highest activity in kidney, liver and plasma extracts, but STX binding activity could not be identified in skeletal muscle, eggs, stomach or skin. The appearance of the activity in plasma would make sense if the STXBP acts as a transport mechanism for an unidentified ligand. In order to compete for STX binding, the endogenous ligand would need to have either similar structural characteristics or an appropriate surface charge that allow binding at the same site on the *B. marinus* STXBP. LC-FD evidence from this study did not identify any known PST derivatives in MeOH or AOAC extracts (see Chapter 3) of *B. marinus*. Based on this LC-FD evidence it is likely that the STX-inhibitory component has a different structure to STX but with similar binding motifs. The clear lack of binding activity in the sodium channel assay further substantiates that the endogenous ligand is dissimilar to STX and may in fact bind to the STXBP by a different mechanism. Structurally, STX consists of a

purine and pyrrole ring system with two highly reactive amino groups at either side of the molecule (see Fig. 5.16) and some of these components are reminiscent of other ubiquitous endogenous compounds such as adenosine or its antagonist theophylline (Fig. 5.17), and even steroid moieties of cardiac glycosides which have been isolated from *Bufonid* toads (see Fig. 5.17).

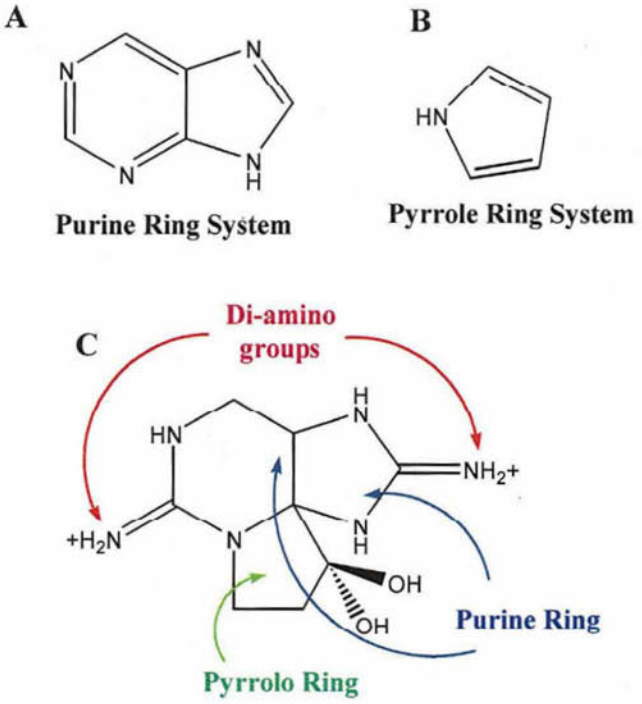

Figure 5.16 Structure of purine (A) and pyrrolo (B) ring systems which form part of the STX parent structure (C). Pyrrolo, purine and diamino groups on the STX backbone structure are illustrated.

Assay guided fractionation of the MeOH extracts from *B. marinus* viscera resulted in the isolation of an STX inhibitory component at 20-40% MeOH with the highest activity observed in the 30% fraction. This data implies that the endogenous component is likely to be polar. The positively charged di-amino groups on the STX molecule (see Fig. 5.16) are thought to bind exposed negatively charged carboxylates on the extracellular surface of the VGSC (Lipkind and Fozzard, 1994). If the endogenous polar ligand isolated during this chapter binds to the toad STXBP in the same manner as STX binds to the VGSC, we may expect that the molecule also consists of at least one positively charged guanidinium group. However, even at the highest concentrations tested, the

endogenous component did not completely displace STX activity (see Fig. 5.4), which suggests that the isolated ligand is acting as an allosteric inhibitor rather than a direct competitor at the STX binding site. If this is the case, the endogenous component is more likely to be binding at an alternative site on the STXBP in the toad, changing either the protein conformation, electrostatic charge, or by partially obscuring the STX binding site. It is conceivable that the *B. marinus* endogenous component is a polar hydrophilic analog of a cardiac glycoside such as marinobufagenin or bufotalin (see Fig. 5.17) particularly when *Bufonis* venom consists of a variety of these compounds (Gowda et al., 2003; Krenn and Kopp, 1998). A protein, such as the STXBP, may act as a transport molecule for these proposed glycosidic compounds, and thereby protect the animal from the potentially fatal effects of these molecules.

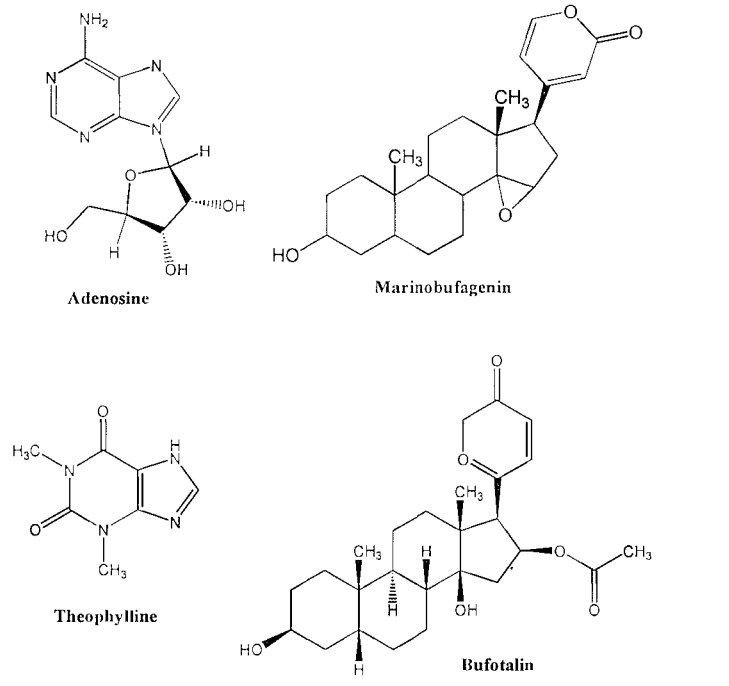

Figure 5.17 Chemical structures of the nitrogenous adenosine and theophylline and cardiac glycosides: marinobufagenin and bufotelin.

The next logical step for the examination of the active polar endogenous fraction from *B. marinus* MeOH extracts would be thin-layer chromatography (TLC). Combined with a variety of chemical sprays such as ninhydrin (for amino acids and glycosides), fluorescamine (for primary or secondary amines, peptides and sulfonamides) or antimony pentachloride (for vitamins, terpenes and steroids),

TLC results may highlight the primary structural moieties of the unidentified ligand from *B. marinus*. Structural elucidation by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) was beyond the scope of this thesis but should also be pursued in future studies and may unravel the biological role of STXBPs in *B. marinus*.

5.4.4 Purification and Partial Sequencing of STXBPs from B. marinus

Several preliminary chromatographic purification trials were conducted during the course of this research which led to the relatively simple two-step protocol involving the strong AEX Q-Sepharose and SEC on Superdex 200HR. Size estimation of the STXBP from *B. marinus* plasma by SEC and subsequent SDS-PAGE was performed by constructing a standard curve following the elution and reported retention of protein standards. Based on SEC, STXBP size was estimated at 87 ± 5 kDa which confirmed previous crude estimations between 50-100 kDa (see Chapter 3). SDS-PAGE of purified STXBP resulted in an estimate of 93 ± 3 kDa which was within the same order of magnitude as previous estimates from *B. marinus* (Chapter 3) and also SXPN from *R. catesbeiana* which was approximated at 74 ± 8 kDa following preliminary purification (Mahar et al., 1991), 89 kDa by final separation (Li and Moczydlowski, 1991) and 91kDa following molecular cloning (Morabito and Moczydlowski, 1994). Amino acid sequencing of *B. marinus* STXBP by MS manual sequencing resulted in 3 distinct peptides (see Table 5.6). Degenerate primers were designed from back-translation of these peptides incorporating codon frequencies for *B. marinus* to enhance the probability of amplification (see Table 5.7). Initial PCR studies with non-redundant combinations of devised primer pairs and cDNA from toad liver were unsuccessful but an increase in Mg^{2+} concentration from initial 1.5 mM to 6 mM and reduction in annealing temperature from 60 °C to 48 °C resulted in the amplification of several products. One drawback to these necessary changes to the PCR conditions is that an increase in Mg^{2+} concentration and reduction in annealing temperature can reduce the specificity of the reaction, resulting in the amplification of non-related sequences. However, prior database comparisons of degenerate primer pairs revealed no matches to any known gene or protein sequences. Subsequent cloning and nucleotide sequencing produced more than 50 sequences, of which only 37 contained corresponding inserts from ligation and

transformation reactions. Almost 40 % of these sequences were homologous to both TFs and SXPN suggesting that the STXBP from *B. marinus* may also be related to these proteins (see Appendix 4). Interestingly, some of these fragments revealed homology to albumin which is known to transport cardiac glycosides in vertebrates (Schoner, 2002). However, given the dominance of albumin in the plasma of all vertebrates it not clear whether its presence is relevant to the STXBP and may be determined following isolation of gene specific sequence in future studies.

Following a series of chromatographic editorial analyses of these partial fragments, 15 nucleotide sequences were obtained, six of which were related to iron binding proteins such as TF as determined by BLASTX analysis (see Table 5.8). Protein sequence alignment of bullfrog SXPN (RC-SX) and a variety of TFs including *Xenopus laevis* serotransferrin (XL-ST), *Gallus gallus* ovotransferrin (GG-OT; chicken) and human serum TF (HS-ST), lactotransferrin (HS-LT) and melanotransferrin (HS-MT) and the translated ORFs of five partial sequences revealed up to 55% sequence identity to TF and SXPN in some instances (Fig. 5.15). The most homologous partial sequence obtained, AR_S08, consisted of and aligned with six of the conserved cysteine residues in a region of the TF and SXPN corresponding to the C-lobe and thought to form disulphide bonds (see Fig 5.15). This alignment also suggests that AR_S08 is highly likely to be a partial fragment of *B. marinus* TF due to the presence of all four of the highly conserved amino acid residues known to be involved in the Fe^{3+}/ HCO_3^- binding site in the C-Lobe of TFs, all of which are substituted in bullfrog SXPN. This likelihood does not preclude the possibility of both TF and SXPN being co-amplified by gene specific primers designed from this sequence in future applications. For instance, primers designed from highly conserved regions may facilitate the amplification of toad transferrin while primers designed from less conserved regions may amplify SXPN from a toad cDNA library. The elucidation of both proteins from the cane toad may greatly assist in future examination of the relationship between TF and SXPN in this species, particularly as *B. marinus* transferrin has not yet been elucidated. Several other partial sequences were also amplified from toad cDNA during this study with varying degrees of homology to TF and SXPN, the relevance of which may become clear in future analyses.

5.5 Conclusions

The results covered in this chapter revealed that *B. marinus* STXBP is very similar to SXPN from *Rana catesbeiana* (see Table 5.9), having a similar size, tissue distribution and presence at all life stages. This is the first study to report that STXBP levels are down-regulated in the cane toad in the cooler months of the year and this may correlate to biological function. Another interesting outcome of this research was the appearance of an endogenous component in MeOH extracts of *B. marinus* that inhibited [^{3}H] STX binding to cane toad STXBP extracts. This component was identified as a polar endogenous ligand dissimilar to known PSTs or TTX and did not affect STX binding activity from other species. Future studies will focus on the structural elucidation of this component and may provide vital clues to the true function of STXBPs in the toad. Also in this chapter, purification of STXBP from *B. marinus* plasma was accomplished by a two-step chromatographic strategy involving anion exchange and size exclusion chromatography. Subsequent partial nucleotide sequencing of this STXBP by degenerate PCR and cloning revealed that several amplified fragments from the cDNA isolated from *B. marinus* liver were homologous to both bullfrog SXPN and a variety of TFs. Gene specific primers designed from these partial fragments may facilitate the isolation and sequencing of the full length STXBP and or serum TF from *B. marinus*.

Table 5.9 Comparison of characteristics between saxiphilin from *Rana catesbeiana* and STXBP from *Bufo marinus* as determined during this chapter.

Factor	Saxiphilin from *Rana catesbeiana*	STXBP from *Bufo marinus*	Reference
Size estimation	91 kDa	93 kDa	(Morabito and Moczydlowski, 1994)
Tissue distribution	All tissues	All tissues	(Mahar et al., 1991)
Life stage	Embryos, Adults	All	(Mahar et al., 1991)
Protein sequence similarity	Transferrins	Transferrins Saxiphilin	(Li and Moczydlowski, 1991)
Ligands	PSTs	PSTs, polar endogenous ligand	(Llewellyn and Moczydlowski, 1994)

5.6 Special Acknowledgements

Dr. Janice Lough from AIMS provided the temperature records which were obtained from the Australian Bureau of Meteorology. Dr Mike Hall provided many pieces of equipment including the Superdex 200HR column, Gilson fraction collector and additional protein related items. Random hexamer, 18S (eukaryote), USP and RSP oligonucleotides were kindly provided by Dr. Kate Wilson and Mrs. Jennifer Swan from the AIMS aquaculture group and both facilitated the research through technical advice, discussions and software training. I would particularly like to acknowledge Dr. Rick Willis and Mr. Jason Doyle and his family all who provided and assisted with toad collections. Mrs. Deborah Pergolotti kindly allowed the use of her cane toad photographs which were incorporated into a diagram of the toad life-cycle (Fig. 5.2).

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CHAPTER 6

General Conclusions and Perspectives

6.1 Research Overview

This thesis has described a multi-faceted experimental approach to the study of STXBPs, drawing heavily upon the disciplines of biology, pharmacology, chemistry, biochemistry and molecular biology to explore the possible role of this unique suite of receptors.

The first and most critical step was to develop a robust and reliable STXBP detection method for utilisation in the subsequent studies on the biology and function of STXBPs. Following extensive trials of a variety of methodologies, a radio-receptor filtration assay incorporating a protein binding membrane of mixed cellulose esters was developed and optimised (see Chapter 2). This assay out-performed all other methods trialled including previously published assays due to the following attributes:

- Robust to the effects of salt, pH and charge
- Capable of producing accurate and reliable data
- Consistently low background
- High cost-effectiveness
- Medium to high throughput
- Universal detection of STX binding activity from biological extracts
- Highly sensitive and selective to PSTs

An extensive phylogenetic survey of STX binding activity and PSTs in more than 1000 individuals representing 203 species across 11 phyla, collected from the marine, terrestrial and freshwater habitats was conducted (Chapter 3). This survey validated the use of the MCE protein binding assay not only as a primary screening tool but as a sensitive assay for the pharmacological characterisation of STX binding sites in biological extracts. During these studies 8 novel STXBPs

were discovered extending the known phylogenetic range of these proteins to include an Antarctic dwelling gastropod (*Laternula elliptica*) and the temperate onychophoran, *Epiperipatus rowelli*. In addition to STX activity, TTX binding activity was observed in extracts of the marine puffer, *Torquiguener pleurogramma* which was deemed to be a likely relative of the puffer fish saxitoxin and tetrodotoxin binding proteins (PSTBPs) previously isolated from *Fugu pardalis* (Yotsu-Yamashita et al., 2002). The present study identified for the first time, co-occurrence of STXBPs and its ligand (STX) in individual animals (the xanthid crab, *Lophozozymus pictor*). If STXBPs are a significant line of defence against PST intoxication, then both protein and ligand may be expected to co-occur, so the lack of STX derivatives in other novel STXBP containing animals did not unequivocally support this hypothesis.

It became clear that exposure to levels of PSTs may be an environmental cue for up-regulation of the STXBPs. On this basis, a known PST "Hot Spot", Cooke Point in Port Hedland, Western Australia (Negri and Llewellyn, 1998), was selected for further examination in Chapter 4. Unambiguous evidence of PSTs in 87 % of benthic animals collected from this site confirmed widespread exposure and one crab species, *Lophozozymus octodentatus,* demonstrated saturable STXBP activity. In addition to the discovery of another novel STXBP, the research from Port Hedland represents the most extensive PST survey conducted in tropical Australia to date, and resulted in several fascinating discoveries including:

- identification of three new macro-algal sources of PSTs
- discovery of 4 novel bivalve PST vectors
- the first report of saxitoxin in the tissues of the octopi
- confirmation of PST biomagnification in benthic marine food web

In chapter 5, the terrestrial amphibian, *Bufo marinus*, was chosen as a model system for an in depth analysis of STXBPs. STXBP levels in the toad were ubiquitous across all life stages and within all tissues with the exception of the venom glands, and reflected previous reports of saxiphilin distribution in the bullfrog *Rana catesbeiana*. Interestingly, a positive correlation was demonstrated

between environmental temperature and yields of STX binding activity in toad plasma over two years. Finally, the STXBP isolated from *B. marinus* plasma was successfully purified, revealing an estimated protein size of 93 kDa and peptide sequence which facilitated degenerate PCR experiments. Cloned STXBP-specific fragments of cDNA from toad liver were sequenced and revealed homology to both saxiphilin from *R. catesbeiana* and a variety of transferrins (see Chapter 5).

6.2 The biological role of STXBPs

The general hypothesis of this thesis was that STXBPs act as a defence against STX intoxication. Clearly the ability to bind STX and many of its derivatives with high affinity (Chapter 3) and evidence of co-occurrence of the mobile receptor and ligand in some crab species (Chapter 3, 4) supports this hypothesis. However, additional evidence of an endogenous ligand of the STXBP in *B. marinus* (Chapter 3, 5) and low occurrence of STXBPs in invertebrates of high PST exposure (Chapter 4) does not. A clear relationship between animals which contain STX binding activity is yet to be identified, and to date activity has only been demonstrated in representatives from ectothermic vertebrates and invertebrates. What then is the biological role of STXBPs?

No clear relationship between the presence of PSTs and the expression of STXBPs was established in animals collected from QLD, the central GBR and Tallaganda state forest. Only one Xanthid crab, *L. pictor*, a known vector of PSTs and TTX (Tsai et al., 1995) was identified with proteinaceous STX binding activity and up to 44 µg STXeq/ 100 g tissue in AOAC extracts (see Chapter 3). Interestingly, STX binding activity has been previously reported in extracts from *L. pictor* specimens collected from the GBR, however the co-occurrence of STX was not (Llewellyn, 1997). If STXBPs are a critical line of defence against PST intoxication, then both protein and ligand would be expected to co-occur more often than was observed in this study.

It was postulated that exposure to PSTs may be an environmental cue for up-regulation of the STXBPs. On this basis, a known PST "Hot Spot", Cooke Point in Port Hedland, Western Australia (Negri and Llewellyn, 1998) was selected for further examination. Unambiguous evidence of PSTs in 87 % of benthic animals

collected from this site confirmed exposure, however as was observed in Chapter 3, only one crab species, *L. octodentatus,* was also identified with STX binding activity (see Chapter 4). In contrast to *Lophozozymus spp.* other intertidal invertebrates from Port Hedland lacked STX binding activity, strengthening the case against toxin defence as a likely biological role of all STXBPs. Despite these findings, it is possible that different structural classes of STXBPs exist in different species, some of which may include biological protection from PSTs. For instance, it is quite possible that the STXBPs identified in the xanthid crabs is akin to the tetrodotoxin binding proteins isolated from the puffer-fish *Takifugu niphobles* (Matsui et al., 2000) and crab *Hemigrapsus sanguineus* (Nagashima et al., 2002) or to the PSTBPs from *F. pardalis* (Yotsu-Yamashita et al., 2002) both of which are thought to have a detoxification role in the animals which produce them. These possibilities can only be determined following full amino acid sequencing and functional expression of STXBPs from individual species.

Based on size estimation and protein sequence homology from Chapter 5, it seems likely that the STXBP from *B. marinus* is related to saxiphilin from *R. catesbeiana,* which has been functionally expressed and was homologous to the transferrin family of iron binding proteins. However, in contrast to bullfrog saxiphilin, *B. marinus* STXBP did not bind to the Heparin sepharose affinity column (Robertson 2003, unpublished data) and had an affinity for neoSTX (Ki = 5.6 ± 0.2 nM; Chapter 3) that was almost 10 times higher than that reported for *R. catesbeiana* saxiphilin (60 nM; (Mahar et al., 1991)). These differences suggest that even if the two proteins have similar amino acid sequences, they may be folded differently changing either the surface charge of the protein or exposure of the STX binding site. Structural elucidation by either NMR or X-ray crystallography will allow accurate modelling of ligand interactions at the STX binding site of the two proteins.

An interesting outcome of this research was the appearance of a polar endogenous ligand in MeOH extracts of *B. marinus* that competed for [^{3}H] STX binding to cane toad STXBP (Chapter 5). While the true nature of the STX inhibition needs to be examined further to determine whether the endogenous ligand is specifically binding to the toad STXBP or simply acting as an allosteric inhibitor, the

appearance of this component is intriguing and may reveal the biological function of STXBPs in this species. No other MeOH or DCM extracts from other species had any effect on STX binding activity to their respective STXBPs or to the *B. marinus* STXBP preparation (Chapter 3) which suggests that the component is unique to this animal. Conversely the endogenous MeOH extract and active C18 fractions from *B. marinus* did not affect STX binding activity from other species (Chapters 3, 5) which may also suggest that cane toad STXBP is quite different from the STXBPs of other species. This data also confirms that assay interference by this component is unlikely.

The biological role of STXBPs including saxiphilin remains a mystery but there is now a wealth of data from this study that has provided several clues and exciting new avenues of investigation that may unravel the true function of these proteins.

6.3 Research Applications

Clear applications of STXBPs include the development of sensitive monitoring devices and analytical tools for the detection of PSTs from fresh water supplies and shellfish. In a functionally expressed form, STXBPs would be accessible in large quantities and so could be used as substrates to ELISA assay formats, bonded to a variety of matrices to form affinity columns which could rapidly capture or purify STXs from environmental samples. Further incorporation of STXBPs with different selectivity for PST derivatives could further enhance these applications by targeting specific toxin analogues.

STXBPs could also be used as STX-targeted plasma absorption molecules in medical applications to treat PST intoxication. Likewise, the development of an antidote would be a great advantage, particularly in Asia where PSP incidence is highest. Currently there is no antidote to PSP and the only medical intervention available to patients is artificial respiration.

The discovery of new sources and vectors of PSTs in tropical Australia is an important issue relating to fisheries regulations and monitoring. For instance, the wild catch octopi fishery in Western Australia does not require routine PST monitoring as is required in the shellfish industry. This is of particular concern

given the high levels reported in octopi collected during this study and a variety of related *Octopus spp.* are targeted for domestic and export product. A clear application of this research would be to develop regulatory or routine monitoring strategies to avoid contaminated product reaching consumers with minimal impact on fisheries practice.

6.4 Future directions

Despite its many merits, one significant drawback to the MCE protein binding assay (Chapter 2), which also applies to the centipede (Llewellyn et al., 1998) and Dowex assays (Mahar et al., 1991; Moczydlowski et al., 1988), was the reliance on tritium labelled STX, which is becoming increasingly difficult to obtain, requires specialist radio-isotope facilities and training, appropriate disposal and is expensive. An alternative fluorescent label, perhaps utilising the phenol ring moiety of the newly identified GC-toxins (Negri et al., 2003) would greatly improve the accessibilities of these methods for monitoring of PST levels in fresh-water sources and shellfish, and particularly to shellfish farmers who would generally lack access to specialist laboratories. The development of a fluorescently labelled STX derivative was well beyond the scope of this thesis, but would be a worthy investigation with many commercial and research applications.

Other interesting directions can be taken from the foundations that this thesis research has provided. For instance, it is now clear that select species of amphibians, reptiles, fish, arthropods, onychophorans and molluscs contain STXBPs, so an important next phase of this work would be to purify and obtain full length cDNA sequence from each species so that a molecular comparison can be made. This data could clearly classify each soluble STXBP as a transferrin related protein as was observed for saxiphilin *R. catesbeiana* (Li and Moczydlowski, 1991; Morabito and Moczydlowski, 1994) and is likely in *B. marinus* (Chapter 5), a PSTBP related protein such as that reported in *F. pardalis* (Yotsu-Yamashita et al., 2002) and likely in *T. pleurogramma* (Chapter 3), or as a completely unrelated group of proteins. Once this information is gathered a true relationship between the proteins of different species can be identified and may

clarify the role of STX as a ligand or implicate vastly different biological roles in each instance.

Clearly the identification of an endogenous ligand in MeOH extracts of *B. marinus* (Chapter 3, 5) should be followed up with purification and structural studies using MS and NMR techniques. The structural identification of this component would not only provide clear evidence of an alternative role of STXBPs in the toad but may also explain the high affinity binding capability of STX to the protein.

Future directions for PST related research would include a complete survey of Cooke Point and other related tropical sites to identify the dominant source and origin of PSTs in the benthic food web. Additional aspects to follow up in this area include:

- Structural verification of PSTs in macro-algae
- Chemical extraction and analysis of epi-plants on macro-algae
- Biochemical pathways involved in PST production by macro-algae
- Extent of food web contamination by PSTs at Cook Point
- Sediment, cyanobacterial and microbiological analysis.

6.5 Additional Outcomes of this Research

In addition to published papers and manuscripts in preparation stated above, several additional outcomes directly and indirectly related to the thesis research are stated below:

- Published collaborative paper and presented poster of research incorporating LC-FD and radio-receptor methods used during this study. My personal role in the collaboration involved crab extraction, assay and assistance with preliminary LC-FD analysis in addition to manuscript preparation and poster preparation.
    - Llewellyn, L.E., Dodd, M.J., **Robertson, A.**, Ericson, G., de Koning, C., & Negri, A.P. (2002) Post-mortem analysis of samples from a human victim of a fatal poisoning caused by the xanthid crab, *Zosimus aeneus*. *Toxicon*, **40**, 1463-1469.

- **Robertson, A.**, Llewellyn, L.E., Dodd, M.J., Ericson, G., de Koning, C., & Negri, A.P. (2002) Forensic analysis of a victim of paralytic shellfish poisoning mediated by the Xanthid crab, *Zozimus aeneus*. Poster Presented at 6th Asia-Pacific Congress on animal, plant & microbial toxins, Cairns, July 2002.

- Collaborative paper accepted incorporating receptor assay, LC-FD and LC-MS methods developed and used during this research. My specific role included performing receptor assays, LC-MS analysis and manuscript preparation.
  - Rapala, J., **Robertson, A.**, Negri, A. P., Berg, K. A., Tuomi, P., Lyra, C., Lahti, K., Hoppu, K., Lepistö, L. First report of saxitoxin and associated human health effects in lakes of Finland. *Journal of Environmental Toxicology*, 26pp (accepted 1st Nov, 2004).

- A comprehensive study on the transport of a variety of PST derivatives (STX, dcSTX, GC 1-3 and GTX 1-4) across chemical hydrophobic and artificial phospholipid layers was conducted incorporating permeability and PAMPA assays with automated LC-FD methodology. My role included complete experimental design, method development, experimental work, data analysis and manuscript preparation.
  - **Robertson, A.**, Motti, C.A., Llewellyn, L.E., Negri, A.P. (2005) Assessment of passive trans-cellular permeability of saxitoxin across hydrophobic and phospholipid membranes. Target Journal: *Environmental Health Perspectives* (in preparation).

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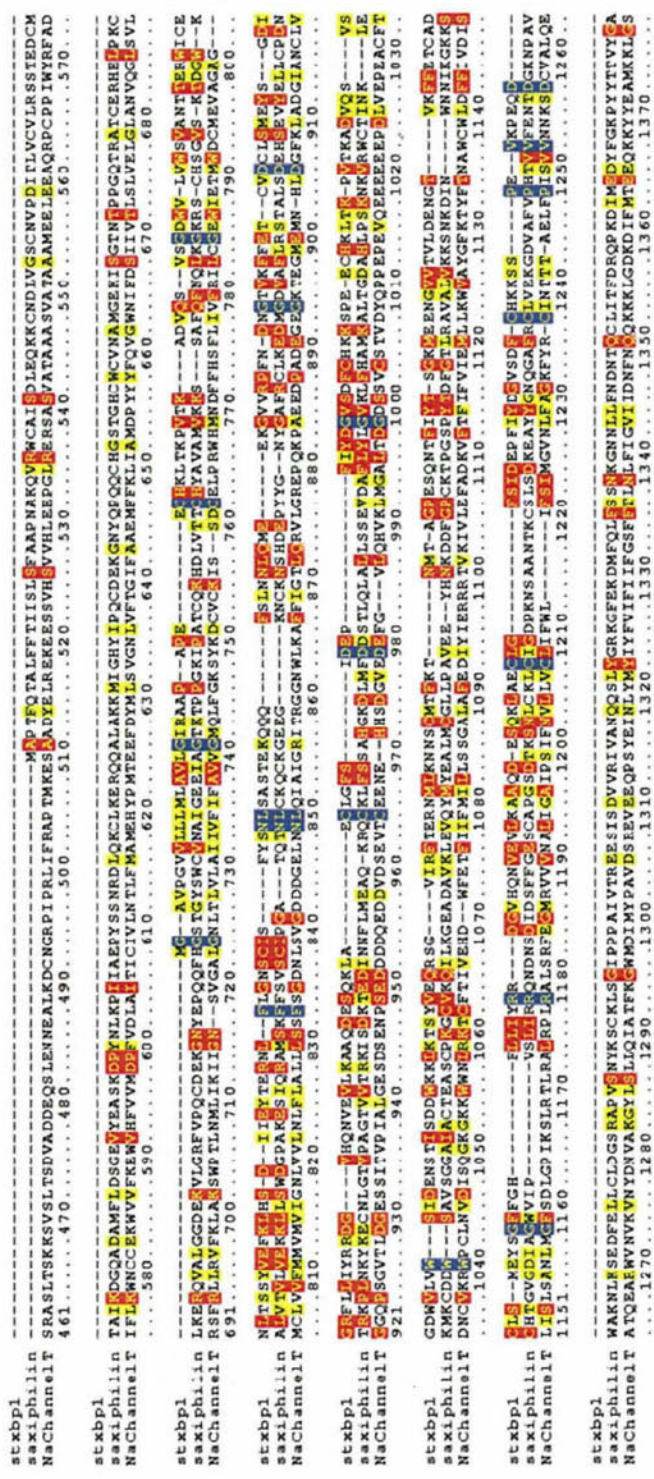
Protein Sequence Alignment of Known Saxitoxin Binding Proteins



Most homologous region of a multiple sequence alignment of the three types of saxitoxin binding proteins; saxitoxin binding protein 1 from the *Takifugu pardalis* (stxbp1), saxiphilin from *Rana catesbeiana* (saxiphilin), and sodium channel sequence from *Takifugu pardalis* (NaChannelT). Sequences obtained from SwisProt + SpTrEMBL databases and aligned with ClustalW. Red=Identical; Yellow=similar; Blue=conserved.

APPENDIX 2

Species collection for phylogenetic survey of sources of STXBPs and PSTs from QLD and NSW sites

ID	Species	Common Name	Local.	Habitat	N	Extract Type
		Vertebrates				
		Fish				
1	*Choerdon schoenlenii*	Black spotted tusk fish	GBR	M	5	P
2	*Tylosaurus crocodilus*	Crocodilian long-tom	GBR	M	5	P
3	*Aethaloperca rogaa*	Red mouth groper	GBR	M	5	P
4	*Caesio cuning*	Fusilier	GBR	M	5	P
5	*Lutijanus carponotatus*	Stripy sea perch	GBR	M	5	P
6	*Lethrinus reticulatus*	Reticulated emperor	GBR	M	5	P
7	*Lethrinus fletus*	Red finned emperor	GBR	M	5	P
8	*Plectropomus leopardus*	Coral trout	GBR	M	5	P
9	*Lethrinus miniatus*	Red throat emperor	GBR	M	5	P
10	*Acanthocybium solandri*	Wahoo	GBR	M	3	P
11	*Scomberomorus munroi*	Spotted mackerel	GBR	M	5	P
12	*Anguilla anguilla*	Eel	GBR	M	7	P
13	*Gobiodon okinawae*	Yellow goby	GBR	M	6	E
14	*Gobiodon unicolour*	Goby	GBR	M	6	E
15	*Gobiodon histrio erythrospilus*	Goby	GBR	M	5	E
16	*Epinephalus merra*	Honeycomb cod	GBR	M	5	P
17	*Istiblennius dussumeieri*	Rockskipper	GBR	M	6	P
18	*Daccyllus sp.*	Damsel fish	GBR	M	5	P
19	*Amblyeleotris sp.*	Goby	GBR	M	5	E
20	*Torquiguener pleurogramma*	Blow fish	GBR	M	5	E
21	*Oreochromis mossambicus*	Tilapia	GC lake, Qld	F	5	E
22	*Poecilia reticulata*	Guppy	AQ	F	5	E
23	*Gambusia hamiltoni*	Mosquito fish	AQ	F	6	E
24	*Lutjanus argentimaculatus*	Mangrove jack	Ross Creek, Qld	F	5	E
25	*Sillago sihama*	Northern whiting	Tully River, Qld	F	6	E
26	*Philypnodon grandiceps*	Flathead gudgeon	Tully River, Qld	F	4	E
27	*Redigobius bikolanus*	Speckled goby	Ross River, Qld	F	5	E
28	*Periophthalmus argentilineatus*	Silver stripe mudskipper	Ross River, Qld	F	6	E
29	*Melanotaenia splendida splendida*	Eastern rainbow fish	AIMS, Qld	F	5	E
30	*Carrassius auratus*	Nymph	AQ	F	5	E
31	*Carrassius auratus sp.*	Micro-scale comet	AQ	F	5	E
32	*Xiphophorus helleri*	Swordfish	AQ	F	6	E
33	*Barbus tetrazona*	Tigerbarb	AQ	F	6	E
34	*Brachydanio rerio*	Zebrafish	AQ	F	6	E
35	*Paracheirodon innesi*	Neon tetra	AQ	F	6	E

ID	Species	Common Name	Local.	Habitat	N	Extract Type
		Amphibians				
36	*Bufo marinus*	Cane Toad	Townsville	T	40	P
		Reptiles				
37	*Naja kaouthia*	Cobra	VS	T	5	P, V
38	*Naja mossambica*	Taiwan cobra	VS	T	5	P, V
39	*Notechis scutatus*	Tiger snake	VS	T	5	P, V
40	*Pseudechis porphyriacus*	Red-bellied black snake	VS	T	5	P, V
41	*Acanthophis antarcticus*	Death adder	VS	T	5	P, V
42	*Pseudonaja textilis*	Taipan	VS	T	5	P, V
		Ascidians				
43	*Didemnum sp.*	White ascidian	GBR	M	6	E
44	*Diademnum sp.*	Purple-white	GBR	M	6	E
45	*Didemnum sp.*	Orange Red	GBR	M	5	E
46	*Aplidium sp.*	Polyclinidae	GBR	M	5	E
47	*Ascidia sp.*	Ascidian	GBR	M	4	E
48	*Clavelina sp.*	Ascidian	GBR	M	5	E
49	*Aplydium sp*	Brown	GBR	M	6	E
50	*Ciona sp.*	Green	GBR	M	4	E
		Echinoderms				
51	*Nardoa noraecaledoniae*	Sea star	GBR	M	6	E
52	*Pentoginaster sp.*	Biscuit star	GBR	M	6	E
53	*Linckia laevigata*	Asteroid star	GBR	M	6	E
54	*Ophioarachns sp.*	Brittle star	GBR	M	5	E
55	*Diademna sp.*	Sea urchin	GBR	M	5	E
56	*Acanthaster planci*	Crown of thorns	GBR	M	4	E
		Bryozoans				
57	*Aplouso branchia*	Bryozoa	GBR	M	5	E
58	*Reteporllina sp.*	Golden leaf	GBR	M	4	E
59	*Stylopoma*	Bryozoa	GBR	M	5	E
		Arthropods				
		Crustaceans				
60	*Callianassa australiensis*	Yabbie	AQ	F	5	H
61	*Cherax depressus*	Blue Yabbie	AQ	F	5	H
62	*Ceriodaphnia sp.*	Water flea	AQ	F	10	E
63	*Penaeus esculentus*	Green tiger prawn	SE	M	5	H
64	*Penaeus monodon*	Tiger prawn	AIMS	M	5	H
65	*Squillidae sp.*	Snapping shrimp	GBR	M	8	H
66	*Thalamita stimpsoni*	Portunid crab	GBR	M	5	E
67	*Alpheus strenuus*	Shrimp	GBR	M	5	E
68	*Leptodius exaratus*	Crab	GBR	M	6	E
69	*Liomera tristis*	Xanthid	GBR	M	5	E
70	*Cyclograpsus granulosa*	Shore crab	GBR	M	4	E
71	*Portunus sp.*	Swimmer crab	GBR	M	6	E
72	*Macropthalmus setosus*	Sentinel crab	Townsville	M	6	E
73	*Thalamita crenata*	Swimmer crab	GBR	M	7	E
74	*Scylla serrata*	Mud crab	SS	M	5	E
75	*Lophozozymus pictor*	Xanthid	GBR	M	4	E
76	*Atergatis floridus*	Xanthid	GBR	M	4	E
77	*Ranina ranina*	Spanner crab	SS	M	5	E
		Insects				
78	*Ctenolepisma sp.*	Silverfish	Townsville	T	12	E
79	*Calolampra sp.*	Wingless cockroach	Townsville	T	12	E

ID	Species	Common Name	Local.	Habitat	N	Extract Type
80	*Macroplanesthia rhinoceros*	Giant rhinoceros cockroach	AIF	T	11	E
81	*Periplaneta fuliginosa*	Smoky cockroach	Innisfail	T	5	E
82	*Archimantis sp.*	Praying mantid	Ayr	T	6	E
83	*Pseudomantis albofimbrata*	False garden mantid	Townsville	T	7	E
84	*Valanga irregularis*	Domestic cricket	Townsville	T	3	E
85	*Austrosalomona falcata*	Katydid	Paluma	T	4	E
86	*Caedicin sp.*	Katydid	Tully	T	5	E
87	*Extatosoma tiaratum*	Spiny leaf insect	AIF	T	5	E
88	*Megacrania batesii*	Stick Insect	AIF	T	5	E
89	*Ctenomorpha sp.*	Pepermint stick	AIF	T	5	E
90	*Abricta curricosta*	Floury baker	Tully	T	5	E
91	*Macrotristria angularis*	Cherrynose	Townsville	T	6	E
92	*Tamasa tristigma*	Cicad	Townsville	T	6	E
93	*Siphanta*	Green Flatid	Cardwell	T	5	E
94	*Ranatra sp.*	Water scorpion	Townsville	T	6	E
95	*Tectocoris diophthalamus*	Harlequin Bug	Cairns	T	7	E
96	*Heoclisis sp.*	Antlion	Townsville	T	8	E
97	*Pamborus sp.*	Ground beetle	Townsville	T	8	E
98	*Trichosterus sp.*	Ground beetle	Townsville	T	7	E
99	*Penthea vermiculata*	longicorn	Crystal Crk	T	9	E
100	*Pseudotetralobus sp.*	Click beetle	Townsville	T	5	E
101	*Phalacrognathus muelleri*	King stag beetle	Paluma	T	6	E
102	*Pharochilus sp.*	Passalid beetle	Paluma	T	4	E
103	*Anoplognathus sp.*	Christmas beetle	Paluma	T	5	E
104	*Lepidiota noxia*	Flower scarab	Townsville	T	6	E
105	*Lepidiota rothei*	Flower scarab	Townsville	T	6	E
106	*Armenis sp.*	Blowfly	Townsville	T	12	E
107	*Toxorhynchites sp.*	Giant mosquito	Townsville	T	5	E
108	*Scaptia sp.*	March fly	Townsville	T	10	E
109	*Xyleutes sp.*	Wood moth	Townsville	T	4	E
110	*Agrotis sp.*	Bogong moth	Townsville	T	4	E
111	*Cosinocera hercules*	Hercules moth	AIF	T	6	E
112	*Perga sp.*	Spitfire grubs	Townsville	T	7	E
113	*Xylotrupes gideon*	Rhinoceros beetle	AIF	T	5	E
114	*Mastachilus sp.*	Squeekers	AIF	T	5	E
115	*Panesthia laevicollis sauss*	Woodies	Townsville	T	6	E
116	*Acheta domesticus*	Cricket	Townsville	T	8	E
117	*Acrophylla wuelfingi*	Giant walking stick	AIF	T	5	E
118	*Alcides sp.*	moth	Townsville	T	4	E
119	*Trigona sp.*	bee	Townsville	T	3	E
		Arachnids				
120	*Desis sp.*	Marine spider	Townsville	M	3	E
121	*Spinicrus sp.*	Harvestmen	NSW	T	5	E
122	*Colossendeis megalonyx*	Sea Spider	Antarctica	M	5	E
123	*Liocheles waigiensis*	Rainforest scorpion	AIF, QLD	T	15	E
124	*Hadroyche sp.*	Funnel Web	NSW	T	5	
125	*Pholcus phalangioides*	Daddy long legs	NSW	T	6	E
126	*Holoplatys spp.*	Jumping spider	Townsville	T	6	E
127	*Latrodectus hasseltii*	Red back spider	Townsville	T	5	E
128	*Lycosa godeffroyi*	Wolf spider	Townsville	T	5	E
129	*Aname sp.*	Trapdoor spider	Townsville	T	5	E
130	*Eriophora sp.*	Orb weaving spider	Townsville	T	5	E
131	*Araneus sp.*	Orb weaver	Townsville	T	5	E
132	*Phonognatha sp.*	Leaf curler	Townsville	T	5	E

ID	Species	Common Name	Local.	Habitat	N	Extract Type
133	*Achaearanea sp.*	House spider	Townsville	T	5	E
134	*Lycosa furcillata*	Wolf spider	Townsville	T	5	E
135	*Eriophora sp*	Weaver	NSW	T	5	E
136	*Delena cancerides*	Huntsman	Townsville	T	5	E
137	*Typostola barbata*	Huntsman	Ingham	T	5	E
		Chilopods				
138	*Cormocephalus sp.*	Rainforest centipede	Paluma	T	6	E
139	*Ethmostigmus rubripes*	Common centipede	Townsville	T	6	E
140	*Spirostrpus sp.*	Millipede	Townsville	T	5	E
141	*Cyliosoma sp.*	Millipede	Townsville	T	7	E
		Onychophorans				
142	*Epiperipatus rowelli*	Velvet worm	NSW	T	22	E
143	*Phallocephale sp.*	Velvet worm	NSW	T	8	E
		Annelids				
144	*Tenebrio molitor*	Meal worm	AQ	T	21	E
145	*Zophobus morio*	Superworm	AQ	T	15	E
146	*Eudrilus eugena*	African nightcrawler	AQ	T	15	E
147	*I. rubellus*	Red worm	AQ	T	15	E
148	*Eisenia fetida*	Tiger worm	AQ	T	15	E
149	*Erpobdella sp.*	Black Leech	Paluma	F	15	E
		Molluscs				
		Gastropods				
150	*Pomocea bridges*	Golden snail	AQ	F	5	E
151	*Ischnochiton australis*	Chiton	AQ	F	5	E
152	*Haliotis asinia*	Abolone	GBR	F	5	E
153	*Helix aspersa*	Garden snail	Townsville	T	5	E
154	*Helix sp.*	Mottled snail	AQ	T	5	E
155	*Ovula ovum*	Egg cowrie	GBR	M	5	E
156	*Monodonta cabrio*	Mono	GBR	M	6	E
157	*Turbo perspeciosus*	Tubinidae	GBR	M	5	E
158	*Trochus histrio*	Trochus	GBR	M	6	E
159	*Tectus pyramis*	Marine snail	GBR	M	6	E
160	*Turbo argyrostomus*	Mollusc	GBR	M	7	E
161	*Mitra sp.*	Spiral shell	GBR	M	5	E
162	*Diadora sp.*	Diad	GBR	M	4	E
163	*Cantharidus sp.*	Canthar shell	GBR	M	4	E
164	*Phasianella sp.*	Spiral	GBR	M	4	E
165	*Rissoina sp.*	Granular spiral	GBR	M	6	E
166	*Strombus luhuanus*	Abolone	GBR	M	5	E
167	*Cypraea araica*	Stromb	GBR	M	3	E
168	*Phyllidia sp.*	Nudibranch	GBR	M	3	E
169	*Chromodoris sp.*	Nudibranch	GBR	M	3	E
		Bivalves				
170	*Sacostrea amasa*	Rock oyster	GBR	M	5	E
171	*Pecten fumatus*	Scallops	SS	M	5	E
172	*Arca avellana*	Ark	GBR	M	5	E
173	*Barbatia dubia*	Doubtful ark	GBR	M	5	E
174	*Laternula elliptica*	Geoduck	Antarctica	M	3	E
175	*Ennucula sp.*	Nuke	GBR	M	5	E
176	*Pinna bicolor*	Razor shell	GBR	M	5	E
177	*Crassostrea echinata*	Spiked oyster	GBR	M	6	E
178	*Mytilus sp.*	Mussel	SS	M	6	E
179	*Malleus sp.*	Mussel	GBR	M	4	E
180	*Saccostrea amasa*	Oyster	GBR	M	5	E
181	*Hydridella sp.*	Bivalve	RR	F	5	E

ID	Species	Common Name	Local.	Habitat	N	Extract Type
		Cephalopods				
182	*Maori octopus*	Octopus	SS	M	5	H
183	*Sepioteuthris australis*	Squid	SS	M	5	H
184	*Sepia sp*	Squid	GBR	M	5	H
185	*Loligo sp*	Squid	SS	M	5	H
186	*Octopud sp.*	Octopus	SS	M	6	E
		Platyhelminthes				
187	*Artioposthia sp1.*	White flatworm	NSW	T	5	E
188	*Artioposthia sp2.*	Black flatworm	NSW	T	5	E
		Cnidaria				
189	*Sarcophyton sp.*	Soft coral	GBR	M	5	E
190	*Anella sp.*	Soft coral	GBR	M	4	E
191	*Briareum sp.*	Encrusting soft coral	GBR	M	5	E
192	*Nepthea sp.*	Soft Coral	GBR	M	3	E
193	*Cynea sp.*	Jellyfish	GBR	M	5	E
194	*Clavularia sp.*	Sea Fan	GBR	M	4	E
195	*Ellesella sp.*	Sea Fan	GBR	M	3	E
		Porifera				
196	*Eustynstyela sp.*	Encrusting sponge	GBR	M	4	E
197	*Reniochalina stalagmitis*	Bright orange	GBR	M	3	E
198	*F. axinellidae*	Finger sponge	GBR	M	5	E
199	*Rhopaloeides odorabile*	Marine sponge	GBR	M	6	E
200	*Crella incrustans*	Marine sponge	GBR	M	5	E
201	*Acanthella sp.*	Marine sponge	GBR	M	4	E
202	*Dysidea sp.*	Marine sponge	GBR	M	4	E
203	*Lanthella sp.*	Marine sponge	GBR	M	5	E

M= Marine, F=Freshwater, T= Terrestrial
E= Extract, H=haemolymph, P= plasma
GBR=great barrier reef, SS= Seafood supplier, AQ= Aquarium supplier, VS= Venom Supplies SA
NSW= New South Wales, QLD= Queensland

APPENDIX 3

Toxin concentration of extracts from individual crab and octopi species collected from Port Hedland.

Species	ID	Trip[§]	µg STX equivalents/ 100g tissue †		
			NaCh	SXPN	LC-FD
Crustaceans					
Lophozozymus	33	A	11	34	10
octodentatus	34 *	A	163	180	145
	35 *	A	1299	873	1124
	36	A	36	17	22
	49*	B	70	84	68
	50 *	B	317	132	104
	51	B	75	70	61
	52	B	6.7	7.5	8.0
	53	B	17	18	23
	54 *	B	48	59	192
	55 *	B	104	171	120
	56	B	7.8	8.0	-
Atergatis floridus	37 *	A	9875	6980	7890
	38 *	A	11236	10031	9600
	39 *	A	10678	8793	10029
	40 *	A	8598	6542	7680
	57 *	B	8830	5100	7338
	58	B	73	55	78
	59 *	B	9871	9560	8978
	60	B	68	79	72
	61 *	B	547	507	612
	62 *	B	1845	1668	2019
	63 *	B	5682	4765	4300
	64 *	B	209	223	275
Thalamita stimpsoni	41 *	A	109	143	96
	42 *	A	102	-	-
	43 *	A	143	113	120
	44	A	-	2.6	14
	65 *	B	98	93	73
	66	B	12	9.3	18.3
	67	B	2.5	1.1	5.4
	68	B	-	-	-
	69	B	18	11	23
	70	B	14	13	-

Appendix 3 (*continued*)

Species	ID	Trip[§]	µg STX equivalents/ 100g tissue †		
			NaCh	SXPN	LC-FD
	71	B	8.4	10	-
	72	B	45	33	34
Pilumnus pulcher	45 *	A	1088	523	229
	46 *	A	672	289	344
	47 *	A	93	45	73
	48 *	A	148	99	62
	73 *	B	114	76	68
	74	B	29	32	-
	75	B	65	53	72
	76	B	18	14	24
	77 *	B	80	91	76
	78 *	B	93	84	78
Octopi					
Octopus (Abdopus)	29 *	A	210	178	246
sp. 5	30 *	A	72	81	102
	31	A	19	13	29
	32	A	34	29	43
	79*	B	695	578	494
	80 *	B	430	220	254
	81	B	10.8	8.5	2.9
	82 *	B	105	98	84
	83	B	1.5	2.3	-
	84	B	65	50	61

- Below detectable limits

† STX equivalents calculated as described in "Methods' and equated to µg/100g organism.

* Individuals toxicity > 80µg/100g tissue by at least one method and thus exceeding public safe limit.

§Collection trips A and B as detailed in "Methods"

APPENDIX 4

BLASTX results of partial sequences resulting from cloning of degenerate PCR fragments into PGemT-easy vector.

ID	Sequence length*	Best Hit Description†	Best Hit Species‡	E Value§
AR_01	639	Serum albumin	*Rana catesbeiana*	1e-24
AR_02	164	No Hits	-	100.0
AR_03	550	Sero-transferrin precursor	*Xenopus laevis*	2e-29
AR_04	1587	No Hits	-	100.0
AR_05	1110	transferrin	*Ammodytes marinus*	4e-23
AR_06	677	Sero-transferrin	*Mus musculus*	3e-24
AR_07	1115	No Hits	-	100.0
AR_08	1205	Sero-transferrin	*Gillichthys mirabilis*	1e-05
AR_09	1170	Myo-inositol monophosphatase	*Sus scrofa*	5.2
AR_10	688	Transferrin	*Salmo trutta*	1e-26
AR_11	623	Sero-transferrin	*Xenopus laevis*	2e-39
AR_12	628	Sero-transferrin	*Xenopus laevis*	5e-35
AR_13	1210	Serot-ransferrin	*Xenopus laevis*	7e-31
AR_14	1419	No Hits		100.0
AR_15	1305	zgc:85809	*Brachydanio rerio*	7e-15
AR_16	1329	Vitamin-d binding protein	*Gallus gallus*	2e-17
AR_17	1453	Sensor kinase/response regulator	*Streptomyces avermitilis*	0.26
AR_18	1256	Vitamin d-binding protein	*Oryctolagus cuniculus*	4e-11
AR_19	1166	Sero-transferrin	*Gillichthys mirabilis*	6e-10
AR_20	1224	Transferrin	*Oncorhynchus tschawytscha*	5e-24
AR_21	1255	Saxiphilin	*Rana catesbeiana*	2e-17
AR_22	1288	Morphogenetic protein receptor II	*Mus musculus*	2e-08
AR_23	1357	No Hits	-	100.0
AR_24	1251	Hypothetical protein	*Neurospora crassa*	9.6
AR_25	1221	Complement component c6	*Branchiostoma belcheri*	1.8
AR_26	409	No Hits	-	100.0
AR_27	1203	clib99	*Yarrowia lipolytica*	0.46
AR_28	1171	Genomic DNA, chromosome 5	*Arabidopsis thaliana*	0.46
AR_29	1224	cg2839-pa	*Drosophila melanogaster*	0.37
AR_30	1313	Sero-transferrin	*Xenopus laevis*	0.06
AR_31	1557	agcp11251	*Anopheles gambiae*	9.4
AR_32	1055	bal-667.	*Rattus norvegicus*	7e-15
AR_33	900	Sero-transferrin	*Homo sapiens*	1e-19
AR_34	1250	Hypothetical protein	*Oryza sativa*	0.011
AR_35	758	Iron binding protein.	*Chrysemys scripta elegans.*	0.001
AR_36	303	No Hits	-	100.0
AR_37	1297	transferrin	*Marmota monax*	3e-07

* Number of nucleotides
† Descriptions are truncated from BLASTX results performed in BioManager by ANGIS.
‡ Species from which best hit record was obtained
§ E-value

Note: Vector contamination was removed and sequence ends trimmed in Sequencher® prior to BLASTX analysis.