

**Sea cucumber fisheries in the Kingdom of Tonga: regeneration
biology, ecology, and environmental chemistry**

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This thesis is dedicated to my late Great Grandmother - an incredible woman whose memory continues to inspire me each and every day.

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Abstract

The goals of this thesis were to address the environmental and anthropogenic factors which may be influencing sea cucumber decline in the nearshore environment around Tongatapu. Physiology, regeneration biology, ecology, biochemistry and environmental chemistry techniques were used to contribute biologically-meaningful data to the Tonga Fisheries Division, to assist in informing management decisions of this important resource. The regeneration study was undertaken to determine the energetic costs of a traditional fishing method on the fishery species *Stichopus horrens*, and the sustainability of traditional fishing practices. The findings of this research component indicate that *S. horrens* can survive and regenerate organs following their harvest, but that this process comes at an energetic cost. The second component of this research involved the quantification of sea cucumber species and their length-distribution in the nearshore environment of Tongatapu. The results of the ecological surveys indicate that sea cucumber stocks are low in some areas, and that this is relatively unrelated to habitat variables. Finally, trace metal concentrations of sediment and holothuroid tissue samples from Tongatapu Island group were determined and used to conduct ecological and human health risk assessments. Sediments were low in all trace metals except Hg, which exceeded the 'low' trigger values but were lower than the 'high' trigger values. The human health risk assessment identified that consumption of holothuroids from this region may result in exposure to Ni, As, and Zn above regulatory limits.

1. General introduction

Sea cucumbers are Echinoderms in the class Holothuroidea. They have a cylindrical appearance, an anus at one end and a mouth at the other (Lambert, 1997). Sea cucumbers have pentaradial symmetry, an internal skeleton, tube feet which function in both locomotion and feeding, and a ‘leathery skin’ which can be very different in texture, thickness and appearance between species (Jangoux & Lawrence, 1982; Lambert, 1997) (**Figure 1.1**). They are benthic organisms that play key ecological roles in sediment bioturbation and nutrient cycling, and as such they contribute to the productivity of seagrass and reef habitats (Uthicke, 2001a; Eriksson et al., 2012; Purcell et al., 2013).

Sea cucumbers are an important food source for many societies around the world. The dried body wall, known as Beche-de-mer, is a popular food item in China representing one of the four luxury banquet items (Barron et al., 2014) and is widely consumed throughout Asia. In the Pacific Islands, sea cucumbers are exported whole or as Beche-de-mer (Purcell, 2014). They are also fished locally in the Cook Islands, Samoa and Tonga, where the internal organs are harvested from the animals and eaten raw (Drumm & Loneragan, 2005; Eriksson, 2006). As sea cucumbers are able to regenerate internal organs, this local fishing practice is not necessarily fatal to the animals.

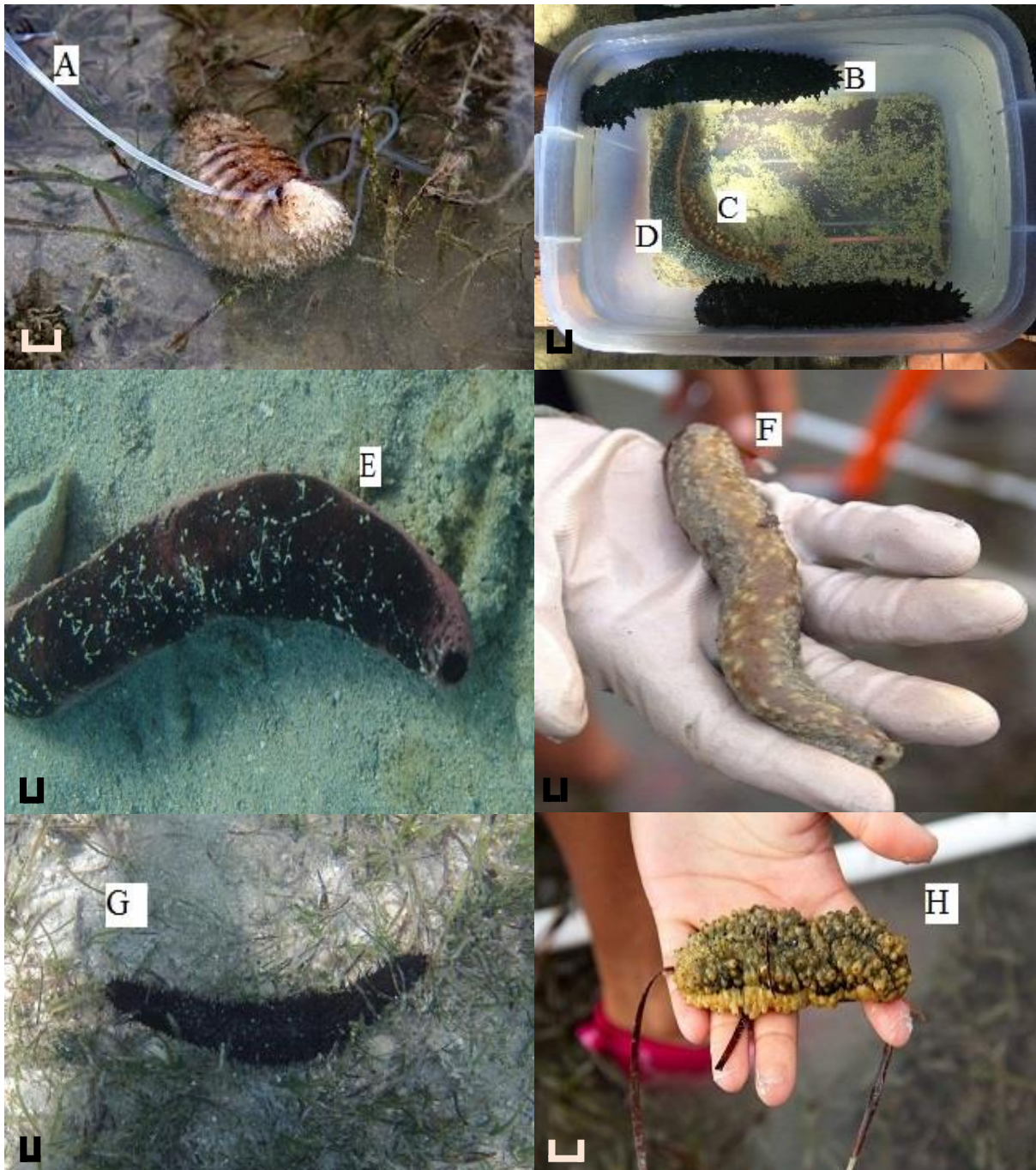


Figure 1.1. Photographs of various sea cucumber specimens encountered in the Kingdom of Tonga. A = *Bohadschia marmorata*; B = *Stichopus chloronotus*; C = *Holothuria hilla*; D = *Holothuria edulis*; E = close-up of *Holothuria edulis*; F = close-up of *Holothuria hilla*; G = *Holothuria leucospilota*; H = *Stichopus horrens*. Photo cred: Sharyn Goldstien and Beth Vanderhaven. The bars in the bottom left corner of each photograph are scales, with the distance between ticks representative of 1 cm.

Over 50% of the human population occupy coastal areas and rely on the ocean as a primary source of protein (Dulvy et al., 2003). However, many seafood sources are under threat due to exploitation and habitat loss causing local, regional and global extinctions of many marine species (Dulvy et al., 2003). For example, exploitation has resulted in the global extinctions of the Steller's sea cow (*Hydrodamalis gigas*), the Horn snail (*Cerithidea fuscata*), and contributed to the local extinction of the Gray whale (*Eschrichtius robustus*) in the Wadden Sea (Carlton, 1993; Carlton et al., 1999; Wolff, 2000; Dulvy et al., 2003). There are also examples of local extinctions resulting from subsistence fishing, such as that of the giant clam, *Hippopus hippopus*, in Tonga (Chesher, 1989). The subsequent diversity loss following extinctions is of direct anthropogenic concern because it results in a decrease in the function and services of an ecosystem (Worm et al., 2006).

Sea cucumber fisheries have typically exhibited a boom and bust cycle, with many populations demonstrating the effects of overexploitation due to commercial fishing (Kinch et al., 2008). Extinction of sea cucumber species at the scale of whole countries has yet to be reported (Friedman et al., 2011), although stocks of *Holothuria scabra* in Malaysia and Papua New Guinea have been described as being close to extinction (Uthicke & Conand, 2005). Local extinctions have been reported in Indonesia, Vietnam, the Philippines, and Egypt (Hasan, 2005; Choo 2008a,b; Purcell et al., 2010), and for all species, extinction risk is greatest for those of highest commercial value (Conand et al., 2014). However, even small populations which may be at risk of extinction are still able to reproduce as long as spawning remains effective (Friedman et al., 2011). When this is not the case, overexploitation puts sea cucumbers at risk of the Allee effect (density limiting reproduction), and it is these two factors (overexploitation and the subsequent Allee effect), which influenced the extinction of the giant clam *Tridacna gigas* in Fiji, Guam, New Caledonia and the Northern Marianas (Wells 1997; Roberts & Hawkins, 1999). Thus, careful monitoring of sea cucumber stocks is required to prevent extinctions at local and regional scales occurring.

1. 1. Commercial export fishery: Tonga

The Kingdom of Tonga is an island archipelago in the Southwest Pacific Islands located in the general area of 21.1333 ° S, 175.2000 ° W. Until 2015, Tonga was among one of six Pacific Island groups that had a sea cucumber fishery open for export, with 82% of exports landing in Hong Kong (Purcell, 2014). In the 1990s commercial fishing became prominent in Tonga, but the fishery collapsed and as a result, a 10-year moratorium on commercial fishing

was implemented in 1996, preventing any sea cucumbers being taken for export (Friedman et al., 2011). Before the collapse of the fishery during this time, the value of the sea cucumber export fishery in 1995 was \$US911, 000 (Ferdouse, 2004), which is a significant income for the Tongan people. Unfortunately, within 2-4 years of the fishery being reopened, it had earned its current classification as ‘depleted’ (Purcell et al., 2013). This resulted in a five-year moratorium on commercial sea cucumber fishing being implemented in 2015 (pers. comm. T. Halafihi, 11.02.2016). Before the implementation of the moratorium in 2015, the value of Tongan sea cucumber exports exceeded that of the Tongan fin-fish fishery (Purcell et al., 2013).

The exploitation of sea cucumbers has resulted in the depletion of high value species such as the White teatfish, *Holothuria fuscogilva* (Pakoa et al., 2009), causing a shift in fishing efforts from high to medium and low valued species (Pakoa et al., 2009). The majority of Tongan sea cucumber exports are now of medium value species such as the Curryfish, *Stichopus hermanni* and the Stonefish, *Actinopyga lecanora* (Pakoa et al., 2009). The shift in commercial fishing efforts from higher to lower value species is concerning because this may result in a clash in fishing interests between traditional fishing (harvesting the viscera and gonads) and commercial fishing (for export).

1. 2. Traditional fishery; Tonga

Stichopus horrens (Lomu in Tongan) is a sea cucumber species of low commercial value (Conand, 2008). In Tonga, this species is fished traditionally by local communities and is an important food and income source for many families. There are two scales of the traditional fishery, the subsistence fishery, and the commercial traditional fishery. For the subsistence fishery for home consumption, the viscera are harvested by making a lateral incision on the mid-dorsal side of the animal and pulling out the internal organs. Any residual sediment is then squeezed out of the intestines. For the commercial-scale traditional fishery, local people walk into the seagrass beds, collect the sea cucumbers in buckets, and harvest the internal organs on the shore (pers. comm. Siola’a Malimali, 18.01.2016). A similar process has been described in Samoa (Eriksson, 2006). The viscera are sold at local markets, and the animals often do not survive this particular fishing method (Eriksson, 2006; pers. obs. Tongatapu 2014 and 2015). However, the animals can survive the method used for gutting the sea cucumbers for home consumption (when they are placed directly back into the seagrass following excision) (Pakoa et al., 2009; pers. comm. Siola’a Malimali, 18.01.2016). In this

thesis, the terms traditional and subsistence fishery are used interchangeably to encompass both of the traditional fisheries (i.e. that for home consumption and the ‘commercial-scale traditional fishery’). The term ‘commercial’ or ‘export’ fishery is used exclusively for describing the industry involved in exporting sea cucumbers for Beche-de-mer.

1. 3. Regeneration

Regeneration is a phenomenon demonstrated throughout the natural world in both vertebrates and invertebrates. Some examples of regeneration include; deer antlers, amphibian limbs, wound healing in axolotls and complete internal organ regeneration in holothurians (Brockes, 1997; García-Arrarás & Greenberg, 2001; Kierdorf et al., 2007; Seifert et al., 2012). The extent to which organisms are able to regenerate is generally a function of their morphological complexity; with regeneration being more extensive in animals at lower levels of complexity than at higher levels (Needham, 1952).

Evisceration, in which an animal ejects its viscera, has been demonstrated in sea cucumbers (Dolmatov et al., 2012). Evisceration of internal organs can act as a predator evasion technique, whereby the predator is distracted by the eviscerated organs and feeds on them while the prey is able to escape (Needham, 1952). Sea cucumbers are also able to auto-eviscerate. Auto-evisceration occurs when the sea cucumber expels its internal organs without any perceived external prompting. This has been demonstrated in *Stichopus regalis*, where auto-avisceration occurs towards the end of summer (Bertolini 1932, cited in Kille 1935). It has been speculated that seasonal evisceration may occur in relation to a decrease in food availability, or as a mechanism to abandon feeding organs which may be full of waste following increased feeding activity in the summer (Byrne, 1985; Fankboner & Cameron, 1985). Extensive regenerative properties have been demonstrated in echinoderms capable of autotomising critical organs (Hsieh, 2012).

Incredibly, sea cucumbers are able to regenerate their internal organs following evisceration or excision. Species in which internal organ regeneration has been demonstrated includes: *Holothuria glaberrima*, *Apostichopus japonicus*, *Holothuria glaberrima* and *Stihopus mollis* (Dawbin, 1949; García-Arrarás et al., 1998; Ortiz-Pineda et al., 2009; Sun et al., 2011). Complete regeneration includes the digestive tract, respiratory trees and gonads, as well as the cuvierian tubules. The cuvierian tubules are sticky white threads expelled from the cloaca following provocation in an attempt to deter predators (Vandenspiegel et al., 2000). Additionally, sea cucumbers are also able to regenerate muscle and nervous systems, and to

heal external wounds (García-Arrarás et al., 1999; Dolmatov & Ginanova, 2001; Dolmatov & Ginanova, 2009).

The process of regenerating the eviscerated organs is often split into three stages: wound healing, regeneration and growth (Needham, 1965 in Kiortsis & Trampusch, 1965; Vandenspiegel et al., 2000; Carnevali, 2006; Dolmatov & Ginanova, 2009). Wound healing is the first step to occur because it prevents both further loss of internal fluid and the invasion of foreign material from the external environment (Needham, 1952). Following wound healing, the regeneration of internal organs occurs as a result of profound proliferation and migration of cells from the remaining intact parts of the animal (Dolmatov & Ginanova, 2009). The rate of regeneration can depend on the functional importance of an organ, the temperature (with higher temperatures corresponding to an increased regeneration rate), and the age of the animal (Needham, 1952; Bai, 1994; Clark et al., 2007). Additionally, the number of times an animal has regenerated can also affect the rate or outcome of regeneration (Needham, 1952). For example, in the flatworm, *Macrostomum lignano*, repeated regeneration resulted in eye loss, outgrowths and skin grooves (Egger et al., 2006). To the best of my knowledge, little is understood of the effects of repeated regeneration in sea cucumbers. It is also possible that regeneration can be affected by trace metals. This has been demonstrated in Crustacea whereby Hg and Cd were shown to produce morphological changes during the regeneration process (Weis et al., 1992).

1. 4. Trace metals as pollutants in the marine environment

Trace metals are elements that are found naturally on Earth, and although they are essential for life processes, they can also be toxic to biota (Giller et al., 1998; Rainbow, 2002; Yadav, 2010; Tchounwou et al., 2012). While the natural levels of trace elements in most areas of the world are relatively low, anthropogenic activities such as refuse burning, construction, fossil fuel burning, electricity generation, agriculture, industry, sewage, and land clearance have increased the concentrations of trace metals in the environment in some locations (Nriagu & Pacyna, 1988; Senesil et al., 1999; Callender, 2003; Gholampour, et al., 2016). Trace metals are often introduced to the coastal environment from rivers, groundwater, atmospheric, and hydrothermal processes (Duce et al., 1991; Carman et al., 2007; Mason, 2013; Tovar-Sánchez et al., 2014). Once in the aquatic environment, trace metals will either partition into sediment or remain dissolved in the water column. (Salomons & Förstner, 1984; Windom et al., 1989; Kowalski et al., 2009). Bioaccumulation, whereby the accumulation of the pollutant in the

tissues exceeds the rate of excretion, can be the result of direct absorption of dissolved metal via the water, uptake of particulate matter, and through the diet (Van der Oost et al., 2003). Trace metal accumulation has been demonstrated in a range of aquatic biota and fish, and are known to have toxic effects on aquatic organisms (Bryan, 1971; Moiseenko & Kudryavtseva, 2001; Wang & Rainbow, 2005; Rainbow, 2007).

The accumulation of trace metals in aquatic biota, can represent a threat to human health via seafood consumption. Cadmium, Pb, As, Hg, Zn, Cu, and Al poisoning can cause a multitude of health effects in humans including vomiting, paralysis, pneumonia, and gastrointestinal disorders (Duruibe et al., 2007). Dietary exposure guidelines and regulations have been published by a variety of organisations including the Environmental Protection Agency (EPA) in the United States, and Food Standards Australia New Zealand (FSANZ). It is important to conduct ongoing monitoring of trace metals in aquatic settings to identify bioaccumulation of trace metals and to identify scenarios where there is risk of toxic exposure to humans.

A study conducted by Morrison and Brown (2003) on sediments and shellfish metal concentrations in and around the Fanga'uta lagoon in Tongatapu showed that trace metals in sediments were present in relatively low concentrations, and that shellfish were safe to eat. The authors identified the necessity for ongoing trace metal monitoring in this region due to continuing changes in this environment, but this remains the last published work examining trace metals in Tonga. An earlier study by Naidu et al. (1991), identified Tongatapu as a site of possible trace element contamination, based on the ongoing industrialisation of the island. However, these authors showed that levels of potentially toxic trace metals (Hg, Cu, Cr, Cd, and Pb) were relatively low and were not a cause for concern (Naidu et al., 1991). In regions such as the Pacific Southwest, where populations rely on nearshore fisheries for a significant proportion of their protein (Zann, 1994), it is important to conduct human health risk assessments to identify risk.

Urbanisation, pesticide and fertiliser misuse, and poor sewage treatment in the Pacific has led to the contamination of aquatic sediments with trace metals (Morrison et al., 2013). There is some evidence in this region to suggest pollutant accumulation in biota, a phenomenon that could compromise local fisheries (Morrison et al., 2013). An ecological and human health risk assessment for trace metals in sediments and sea cucumber tissues from Tongatapu was undertaken in the present study.

1. 5. Thesis: Aims and Hypotheses

This thesis is divided into three data chapters, each with a different disciplinary focus. The first chapter is entitled ‘Regeneration biology: the effects of a traditional fishing technique on the physiology and biochemistry of *Stichopus horrens*’, in which the effects of traditional fishing techniques on the subsistence fishery species *Stichopus horrens* were investigated. The specific aims of this chapter were to fill a biological knowledge gap surrounding the energetic costs of internal organ regeneration on *S. horrens*. The goal of this research was to supply scientific principles to marine management, and investigate how this traditional fishing technique may (or may not) be influencing the sustainability of the subsistence fishery. I hypothesise that internal organ regeneration results in a physiological change in terms of energy metabolism, with resulting alterations in energy stores and mobilisation (e.g. of glycogen, glucose, and lactate). I also hypothesise that wound repair and internal organ regeneration come primarily at the cost of sexual reproduction, and that the magnitude of this cost is dependent on external variables.

The second chapter is ‘The ecological distribution and diversity of holothuroids in the Tongatapu Island group’, in which the ecological distribution of sea cucumbers in the nearshore habitat around Tongatapu were investigated. The aim of this research was to quantify the sea cucumber diversity, richness, and abundance around Tongatapu, and to identify factors which may influence patterns in these variables. I hypothesise that habitat variables alone will not be able to explain the differences in sea cucumber diversity between sites around Tongatapu. Additionally, I hypothesise that there will be some areas in which anthropogenic intervention is required to allow sea cucumber stocks to recover from commercial fishing.

The third chapter is entitled ‘Trace metal accumulation in sediment and holothuroid tissues in Tongatapu’. The aims of this chapter were to identify the concentrations of trace metals in sediments and sea cucumber tissues collected from Tongatapu, and to conduct ecological and human health risk assessments. The goal of this research was to determine whether trace metals in their present concentrations in sediments present a risk to aquatic life, and to determine whether the levels of trace metals in sea cucumber tissues may present a human health hazard. I hypothesise that there is likely to be evidence of anthropogenic trace metal inputs in Tonga, and that it is possible that sea cucumbers may present a health risk for some metals, depending on the quantities in which they are consumed.

The overall goal of this research was to assist the Tonga Fisheries Division in quantifying the factors that may be exacerbating the effects of commercial overfishing, and influencing sea cucumber decline around Tonga. It is hoped that this study will provide scientific information that may be useful when determining appropriate steps for future sea cucumber conservation and resource management.

2. Regeneration Biology: The Effects of a Traditional Fishing Technique on the Physiology and Biochemistry of *Stichopus horrens*

2.1. Introduction

Among the many fascinating characteristics of sea cucumber biology is their capacity for regeneration. While clearly an advantageous strategy, it is associated with costs to the animal. Regeneration has the capacity to affect nutrient storage, behaviour and reproduction (Lawrence 1991; Ramsay et al., 2001). Nutrient storage is affected because regeneration is an additional energetic cost, which can take nutrients, and thus energy, away from growth and reproduction (Barrios et al., 2008). A reduction in energy stores in regenerating echinoderms has been demonstrated in the sea star species, *Stichaster striatus* and *Heliaster helianthus*, in which energy stores in autotomised individuals were significantly less than those in intact individuals (Lawrence & Larrain, 1994; Barrios et al., 2008).

Regeneration can affect reproductive capacity (Ramsay et al., 2001). A change in reproductive capacity associated with regeneration has been demonstrated in many species including the spionid polychaete *Polydora ligni*, the brittle star *Ophiocoma echinata* and the lizard species *Coleonyx brevis* (Dial & Fitzpatrick, 1981; Zajac, 1985; Pomory & Lawrence, 2001). In a population of the sea cucumber *Holothuria parvula*, a low frequency of individuals with gonads was explained as being a reflection of constant regeneration preventing gonadal development (Emson & Mladenov, 1987). The biological impact of regeneration may depend on external factors such as the season in which an animal begins regenerating or is autotomised. For example, in the coral species *Fungia granulosa*, individuals regenerating in spring (the reproductive season) never completely regenerated,

while those regenerating in autumn all completely repaired themselves (Kramarsky-Winter & Loya, 2000). These authors discuss the possibility that in the reproductive season, energy that could be used for regeneration was instead used to maintain reproduction, resulting in a decreased rate and frequency of regeneration in wounded corals in the reproductive season compared to the non-reproductive season (Kramarsky-Winter & Loya, 2000). This evidence indicates that regeneration is likely having metabolic costs which are affecting the capacity for sexual reproduction. It also suggests that the relationship between regeneration and reproduction is complex.

Additionally, behavioural effects of regeneration may reduce an animal's fitness. In the sea star species, *Asterias rubens*, multiple arm losses resulted in decreased feeding activity compared with fewer arm losses, indicating that extensive arm loss may result in a loss of fitness by negatively affecting feeding behaviour (Ramsay et al., 2001). Regenerated appendages are often smaller in size compared with non-regenerated appendages, and this can influence feeding behaviours, reproduction and survival rate (Maginnis, 2006a).

The elevated energetic cost of regeneration can also affect growth (Maginnis, 2006a). Trade-offs between growth and regeneration have been demonstrated in many species. For example; in the stick insect *Sipyloidea sipyilus* leg regeneration resulted in a decreased wing size (Maginnis, 2006b). In the lizard species *Uta stansburiana*, young which were re-growing their tails developed at a slower rate than those which were not regenerating (Niewiarowski et al., 1997), and in arthropods regeneration can cause a lag in the onset of moulting (Maginnis, 2006a). It is likely that reduced growth rates in regenerating individuals are a product of the energy used for growth being diverted to regeneration.

In sea cucumbers, an understanding of regeneration biology is of particular significance. Traditional fishing methods involve the removal of gonads via a body wall incision. The impact of such a manipulation on sea cucumbers will impact the sustainability of the fishery. There is already some evidence that this mode of harvesting has effects on sea cucumbers. A study on *Holothuria leucospilota* in the Cook Islands demonstrated that traditional fishing methods (removal of the organs via a body wall incision) had a negative effect on body weight and behaviour, as well as a possible negative effect on reproduction (Drumm & Loneragan, 2005). It is widely accepted that gonads are the last internal organ to regenerate following evisceration or excision (Kille, 1935; Kille, 1942; Dumm & Loneragan, 2005), and sea cucumbers often only spawn annually. Therefore, if gonads are removed during the

spawning season, sexual reproduction may be delayed for another year (Drumm & Loneragan, 2005).

One of the gaps in sea cucumber knowledge, identified as being imperative to the improvement of fisheries management regimes, is the inadequate data on the biology of these animals (Purcell et al., 2013). Understanding the biology of sea cucumbers is important in preventing future fisheries collapse (Eriksson et al., 2012), and this knowledge could be used to implement more successful management regimes in collaboration with the local community. An example of this exists in Vanuatu, where scientific and traditional knowledge were incorporated to achieve more effective management of the Trochus (*Trochus niloticus*) fishery (Johannes, 1998).

It has been shown that regeneration represents an energetic cost, which may come at the expense of growth and reproduction (Lawrence, 2010). Thus, in examining the impact of regeneration at a physiological and biochemical level, ammonia excretion rate (AER), oxygen consumption rate (MO_2), and glucose, glycogen and lactate concentrations in sea cucumber tissue and coelomic fluid can be informative end-points. Ammonia excretion rate and MO_2 are commonly used as biomarkers to indicate the physiological state of an animal (e.g. Chandurvelan et al., 2012). Oxygen consumption rate summarises the energy expenditure of an organism's life processes, and AER indicates the energetic pathways being used through measuring the amount of waste nitrogen excreted, as a proxy of protein metabolism (Urbina & Glover, 2015). Biochemical measures such as lactate can also be indicative of metabolic costs. Lactate is formed in animals when oxygen consumption is insufficient to meet energy demands and can indicate that energy costs are temporarily higher than fuel flux through aerobic pathways (Berg et al., 2002). Additionally, glycogen and glucose can provide insight into the energetic pathways and the patterns of energy use in organisms, as well as provide support to the results of physiological end-points (Urbina et al., 2014).

The current study sought to address existing knowledge gaps regarding the biology of regeneration in the important sea cucumber fishery species *Stichopus horrens*. This species is an important subsistence fishery in the Kingdom of Tonga, and in other islands throughout the Pacific (See Section 1. 2.). The goal of this study was to increase the knowledge surrounding the biology of this species and the effects of subsistence fishing on regeneration and the physiology and biochemistry of these invertebrates, with the aim of contributing

biologically-meaningful data for the sustainable management of this resource for the benefit of the Tongan people and the marine ecosystem.

2.2. Methods

All regeneration experiments were conducted in the Kingdom of Tonga, at the Ministry of Fisheries in Nuku'alofa. Laboratory studies were conducted in the aquaculture buildings or outside tanks, while field studies were performed in the seagrass opposite the Ministry building on Sopu reef (**Figure 2.1**). The consent of the Tongan Ministry of Fisheries was given to carry out this work, and all work was in collaboration with Fisheries staff.



Figure 2.1. Google Earth image showing the Fisheries department of the Ministry of Agriculture, Food, Forestry and Fisheries in Tongatapu island group in the Kingdom of Tonga. The aquaculture section of the Fisheries Department is where the laboratory study was carried out. Additionally, the field experiment location is also shown on the image. Fisheries Department ($21^{\circ} 7'20.21''S$, $175^{\circ} 13'35.62''W$), field experiment ($21^{\circ} 7'16.98''S$, $175^{\circ} 13'32.75''W$).

2. 2. 1. Experimental set-up

2. 2. 1. 1. Laboratory study

Sea cucumbers were collected from the seagrass bed opposite the Ministry of Fisheries on Sopo reef over a period of five days. A total of 48 animals were collected with an average (\pm standard deviation), wet weight of 36.5 ± 20.7 g and length of 11.4 ± 3.7 cm. Animals were held in two outdoor tanks, supplied with flow-through unfiltered natural sea water. Initially, to create replication, both tanks were divided into 6 sections with wire mesh dividers. However, the wire mesh gaps were too large, allowing animals to move between sections. Consequently, to prevent escape, squares of shade cloth were cut and sewn into bags (approximately 120 cm^2 and ~ 1.3 l volume) using fishing wire (**Figure 2.2**). A rock and a handful of sediment comprising a mix of fine, medium and large ($\sim 0.25 \text{ cm}^2$) grains collected from the shore on Sopo reef, were placed in each bag, and these bags were then placed into the outdoor tanks. Two days before the experiment started, the animals were moved into the mesh bags (8 animals per bag). At all times, a water flow rate of $\sim 3 \text{ l min}^{-1}$ was maintained, and water temperature was constant between 24 and 24.5°C



Figure 2.2. Photograph of one shade-cloth bag sewn together using fishing wire. The bags were tied onto a mesh cage.

In total, this experiment had three treatments; excised, cut without evisceration (sham) and control. Each treatment had a total of 16 animals, consisting of 8 animals placed in one of two bags. One bag of each treatment type was placed in each outdoor tank, in a random manner to avoid any bias. The excised treatment involved making a small (approximately 2 cm long) lateral incision on the mid-ventral side of the body wall with a scalpel. The animals were then eviscerated by pulling the respiratory tree, intestines, and gonads (only 4 animals had visible gonads) out through the incision. The remaining animal was placed inside the appropriate bag. All of the internal organs were removed because this is the fishing practice employed by the local people, and it has been shown that when *S. horrens* eviscerates, the gonads, intestine and respiratory trees are all ejected (Dolmatov et al., 2012). It has also been shown that this species can eviscerate through either the cloaca or a rupture in the body wall and therefore the excised treatment can be used as a proxy for natural evisceration (Dolmatov et al., 2012). For the sham treatment, an external incision was made in the same fashion and in the same place as the excised sea cucumbers, but no evisceration was performed, to provide an indication of wound healing costs. The controls were sea cucumbers that did not receive any treatment except handling and placement into bags. The animals that were collected sometimes budded (divided in half), but for this experiment, no budded individuals were used in the experimental set-up.

For laboratory exposures, two time points were sampled, 6 and 10 days, herein referred to as 6dl and 10dl respectively. These time points were determined by a pilot study. This study consisted of two animals being excised and then dissected 10 days afterwards. At 10 days, one of the animals had regenerated ~ 1 cm of intestine and a small amount of respiratory tree. The other animal had regenerated a full intestine (connected to both the oral and anal openings) and a respiratory tree that was more extensive than the other animal. Due to the second animal having a full intestine at 10 days, it was decided that sampling times of 5 and 10 days should capture the periods over which regeneration was occurring. Unfortunately, there were issues with sustained seawater flow on Day 5, that may have impacted the physiological state of the animals, and so the Day 5 sampling was deferred to Day 6.

On the 6dl sampling, 4 animals were taken from each treatment per tank, so given that there were two tanks, a total of $n = 8$ per treatment were sampled. On the 10dl sampling, all of the remaining animals in the bags were removed, but the number of animals per treatment had increased because many of the animals had reproduced via fission (divided in half to form two new animals). The total number of animals that budded over the entirety of the laboratory

experiment was 14, and in the results, the animals which had budded, and those which had not, were tracked.

2. 2. 1. 2. Field Study

A field study was conducted to investigate if the results gained from the laboratory experiment could be obtained under more natural conditions, and because the process of regeneration can be affected by environmental conditions. Sixteen animals were collected from the same area as the laboratory animals (opposite the Ministry of Fisheries on Sopu reef) one day before the experiment began with a mean (\pm standard deviation) wet weight of 24.9 ± 13.2 g, and length of $9.8 \text{ cm} \pm 2.9$ cm. Overnight they were held in bags in neighbouring sections of the same tank to ensure they were kept in similar pre-experimental conditions. The following day, the treatments were administered (excised to one bag and control to another) with $n = 8$ animals per bag. A sham treatment was not included in this study because sham effects had been investigated in the laboratory experiment. A handful of sediment (the same mix as used in the laboratory study) and a rock were placed in each bag to weigh them down. The bags were placed in a patch of seagrass in front of Sopu reef from where the sea cucumbers were initially collected, out of the way of human and boat traffic. This location is close to fished populations of this species. After 10 days, all animals were taken out of their treatments for measurement of AER, MO_2 , glucose, glycogen and lactate concentrations in the body wall and coelomic fluid. For the experiments measuring MO_2 and AER, a total of 8 animals across a mixture of treatments were run in the respiration chambers at a time. The treatments were mixed to avoid measuring the MO_2 and AER of an entire treatment group at one time during the day, which could have potentially confounded the results. Caged field animals are coded as 10df throughout this chapter. The process of regeneration can be affected by environmental conditions, which is why both field and laboratory studies were conducted. As with the laboratory study, animals in the field study also budded (divided in two).

2. 2. 2. Respirometry

Static, closed respirometry was applied in the current study to measure MO_2 . Animals were weighed (g), and put straight into respirometers. The respirometers were then filled with filtered ($1 \mu\text{m}$) seawater to reduce any microbial activity which may have contributed to oxygen consumption.

Initially, sea cucumbers were placed in 1.75 l custom-built Perspex respirometers, but the MO_2 in these was too slow to allow multiple sea cucumbers to be run within a 24-hour time period. To maintain all measurements in a single day, smaller chambers (0.1, 0.25 and 0.5 l Schott bottles, sealed with rubber bungs) were used for respiration experiments. As often as was feasible, sea cucumbers were allocated to respirometers according to their relative body size.

Each animal was placed inside the respirometer and all air bubbles were removed. Inserted into a rubber bung were two, 3-way taps attached to surgical needles, with a reservoir attached to one tap, and the other tap for extraction of water samples. The oxygen content (mmHg) of the sample was read using an oxygen electrode (Strathkelvin), connected to an oxygen meter which was in turn connected to a PowerLab system that was making continuous recordings. The oxygen electrode was housed in a chamber receiving temperature-controlled water set to the exact temperature of the respirometry water. This ranged from 20.1 to 25.2°C. This set-up is demonstrated in **Figure 2.3**. The respirometers were placed in a seawater bath (a plastic box filled with 1 μm filtered seawater). This bath was not able to be temperature-controlled leading to the differences in experimental temperature.

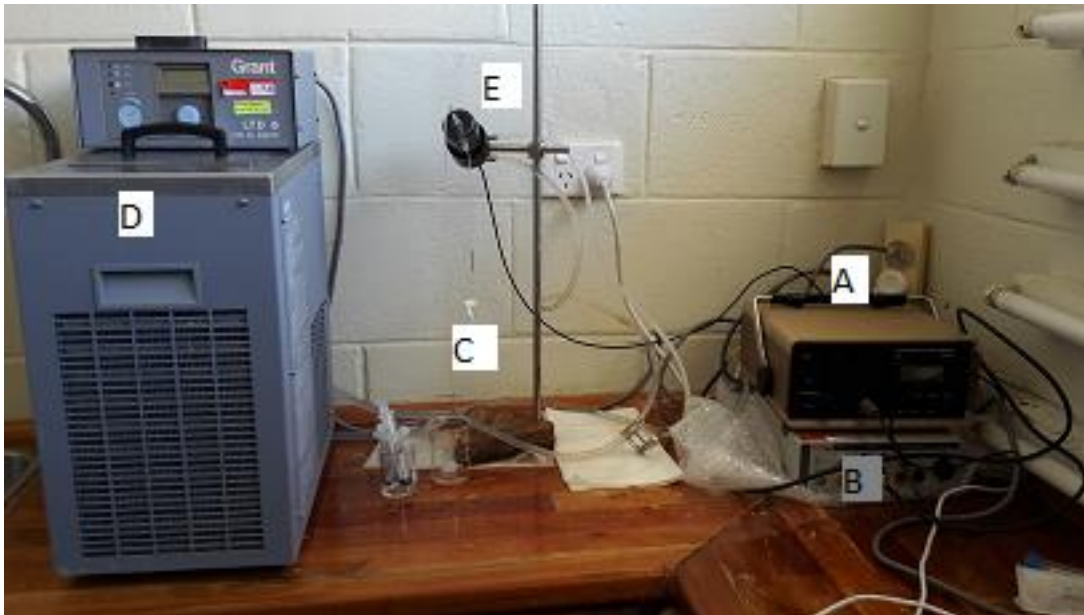


Figure 2.3. Respirometer set-up where; A = oxygen meter, B = PowerLab console, C = valve to insert water sample, D = temperature-controlled water bath, and E = the housing containing the oxygen electrode.

The atmospheric air pressure (millibars) was obtained daily from the weather forecast given by Tonga Meteorological Services (www.met.gov.to). Millibars were converted to mmHg (millimetres of mercury) by multiplying by 0.75. The PO₂ (oxygen partial pressure) at air saturation was then calculated using the Equation 2.1 from Harley (2015):

Equation 2.1

$$PO_{2max} (mmHg) = 0.20946 \times (Pb - P_{wv})$$

Where 0.20946 is the mole fraction or volumetric fraction of oxygen in the atmosphere; Pb is the total barometric pressure (mmHg); and P_{wv} is the water vapour pressure at the experimental temperature (mmHg).

Before each reading, the PO₂ max and zero were recalculated for the oxygen electrode, based on the experimental temperature. The zero solution consisted of 3 ml of 0.01 M sodium tetraborate and a pinch of sodium sulphite. Approximately 1 ml of the zero solution was pushed through the oxygen electrode and left for 2 minutes or until the reading on the oxygen meter had stabilised. The oxygen meter was then re-zeroed accordingly. Once the machine had been zeroed, tap water was pushed through the electrode to remove any remains of the zero solution. Following this, a syringe full of saltwater was pushed through the electrode, and then pulled back out until there was a pocket of air inside the oxygen electrode. After waiting for approximately 2 minutes for the machine to stabilise, the PO₂max was set on the oxygen meter.

Before each experimental water sample was taken, both taps on the respirometer were opened and the syringe was moved up and down several times in order to mix the water inside the respirometer. This was to ensure that oxygen was homogeneously distributed throughout the respirometer. After the water sample from the respirometer was taken, 2/3 of it was inserted into the oxygen electrode. The first time each day that this was done, the PowerLab was connected to determine the length of time it took for the oxygen reading to stabilise. After this time, the remaining water sample was pushed through the electrode and left until the reading had stabilised before the oxygen value was recorded. The times to stabilisation were recorded and kept consistent for all respirometry readings following this. The experimental oxygen readings obtained through this study were in mmHg which were then converted into an oxygen concentration in $\mu\text{moles O}_2 \text{ l}^{-1}$ using Equation 2.2 from Widdows and Staff (2006):

Equation 2.2

$$C(t) = [(Exptl. PO_2 \text{ in mmHg}) / (PO_2 \text{ at air saturation})] \times O_2 \text{ solubility } (\mu\text{moles } O_2 \text{ l}^{-1})$$

Where C(t) is the concentration of oxygen in the water ($\mu\text{moles } O_2 \text{ l}^{-1}$); and O_2 solubility is the oxygen solubility ($\mu\text{moles } O_2 \text{ l}^{-1}$) at the experimental temperature and salinity according to Annex 6 in Widdows and Staff (2006). The experimental salinity was not measured during this study but a salinity of 35.4 ppt was used based on reported information for the source seawater used in this study (Zann et al., 1984; Dassie et al., 2010). Because tabulated salinity values are only in whole numbers, a salinity value of 35 was used. Equation 2.3 was used to calculate MO_2 (Widdows & Staff, 2006):

Equation 2.3

$$\text{Rate of } O_2 \text{ uptake } (\mu\text{moles } O_2 \text{ h}^{-1}) = [C(t_0) - C(t_1)] \times (V_r) \times 60 / (t_1 - t_0)$$

Where t_0 and t_1 are the start and finish times (minutes) of the experiment; C(t) is the concentration of oxygen in the water ($\mu\text{moles } O_2 \text{ l}^{-1}$) at time t; and V_r is the volume of the respirometer (l) minus the animal “volume” (i.e. mass; kilograms).

Respiration values for blank respirometers (chambers without an animal in it) were calculated and were subtracted from sea cucumber respiration values.

2. 2. 3 Ammonia Excretion Rate

Ammonia excretion rate was measured during respirometry experiments. Following an initial sample of respirometry water, water from the respiration chamber was sampled at regular intervals throughout the respirometry experiments. Before each water sample was taken, the contents of the respirometer were syringe-mixed several times to ensure that the water samples were from a homogenous solution. A 1.25 ml water sample was taken when the sample could be assayed immediately. However, it was not always possible for samples to be assayed immediately and for these samples, 1.75 ml of sample was used to fill an Eppendorf tube to the top. These samples were refrigerated until they could be assayed (which was always less than a 24-hour period). The tubes were filled to reduce the loss of ammonia by diffusion as NH_3 , and refrigerated to reduce any microbial activity which could cause a significant change in ammonia.

Using a Pasteur pipette, water was removed from the Eppendorf tubes until the sample was 1.25 ml. The API Ammonia Test Kit for fresh- and salt-water aquaria were used to measure

ammonia concentration. Fifty μL of both test kit solutions were pipetted into the water samples and shaken vigorously. These were left for 10 minutes to allow the colour to develop before being centrifuged until the precipitate had pelleted. The absorbance of the supernatant was read on an Amersham Biosciences Novaspec III visible spectrophotometer at 650 nm using an empty cuvette as a blank.

A standard solution of $1400 \mu\text{g N l}^{-1}$ (as ammonium chloride) was made in seawater filtered to $0.45 \mu\text{m}$, and subsequently serially diluted in seawater to generate an ammonia standard curve. That the standard curve was made up in sample water meant that it accounted for any ammonia in seawater and thus no ammonia blank was needed. An ammonia standard curve was calculated using the concentration of the ammonia standard solutions (x) versus absorbance (y). A linear trend line was fitted on the scatterplot to obtain the value for slope of the line (forced through the origin) which was used to convert the absorbance values to an ammonia concentration in $\mu\text{g l}^{-1}$ of N using Equation 2.4;

Equation 2.4

$$\text{Ammonia concentration in } \mu\text{g l}^{-1} \text{ of N} = (\text{absorbance value/slope})$$

The ammonia concentration in $\mu\text{g l}^{-1}$ of N was then used in Equation 2.5 to calculate a mass-specific amount of ammonia excreted:

Equation 2.5

$$\text{AER } (\mu\text{g g}^{-1}\text{h}^{-1}) = (\text{Ammonia})/((t_F - t_S)/60)/(\text{weight})$$

Where ammonia is the total amount of ammonia in ($\mu\text{g N}$) in the respirometer (concentration from Equation 2.3 x volume of respirometer in L); $t_F - t_S$ is the final experimental time (minutes) – the start experimental time (minutes); and weight is the animal weight in grams.

2. 2. 4. O:N

The AER and MO_2 were used to calculate the average atomic ratio of oxygen used to nitrogen excreted as waste (O:N), in Equation 2.6 (Chandurvelan et al., 2012):

Equation 2.6

$$\text{O:N} = (\mu\text{moles O}_2 \text{ h}^{-1}/16)/(\mu\text{moles NH}_4\text{-N h}^{-1}/14)$$

Owing to missing data, an average O:N was calculated based on the overall means for each experimental group, rather than determining the average of individual O:N within a group.

2. 2. 5. Biochemistry

2. 2. 5. 1. Initial experiments to define methodological procedure

Before going to Tonga, a series of experiments were carried out on New Zealand sea cucumber tissue from *Australostichopus mollis*. These were devised in order to formulate a methodology by which to carry out biochemical analyses while in Tonga. In order to determine the glycogen levels in the tissue or coelomic fluid via a glucose assay, the glycogen must first be broken down into glucose (Berg et al., 2002). There are two commonly employed mechanisms for achieving this: temperature (i.e. heating) or enzymatic conversion. Amylglucosidase is an enzyme which converts glycogen into glucose. Therefore, if amylglucosidase enzyme solution is added to a supernatant (made from the homogenate of a tissue sample), it will convert a certain amount (if not all) of the glycogen into glucose. This solution can then be run through a glucose assay and once the initial glucose level in the homogenate is subtracted, the amount of glucose generated by glycogen breakdown can be determined.

The Sigma product instructions for the enzymatic assay of amylglucosidase in starch use a 50 mM solution of sodium acetate at a pH of 4.5 at 55°C. The enzyme solution itself is reconstituted at a concentration of 0.3 – 0.6 unit ml⁻¹ of enzyme in purified water. Saborowski and Buchholz (1996) used a 0.1 M acetate buffer at pH 4.8 and 20 µl of a 1 mg ml⁻¹ amylglucosidase solution to breakdown glycogen into glucose. Urbina and Glover (2015) incubated samples for glycogen analysis in 100 µl of acetate buffer containing amyloglucosidase (concentration 2 mg ml⁻¹) for 16 hours at 50 °C.

Trial one was an optimisation of supernatant volume, and effectiveness of enzymatic conversion. Supernatant volume is particularly important as the supernatant itself contributes to the absorbance measured, so too much supernatant would obscure the glucose signal, but insufficient supernatant would not contain sufficient glycogen to detect. Three solutions were tested:

1. 10 µl supernatant + 0.5 ml 0.2 M sodium acetate buffer + 40 µl enzyme (amylglucosidase) solution
2. 50 µl supernatant + 0.5 ml 0.2 M sodium acetate buffer + 40 µl enzyme solution
3. 100 µl supernatant + 0.5 ml 0.2 M sodium acetate buffer + 40 µl enzyme solution

Controls (without enzyme) were also run in order to measure baseline absorbance and thus judge the efficiency of enzymatic conversion. After 17 h incubation, 10 µl of each solution were run through a glucose assay. Unfortunately, the results did not give a definitive outcome because the concentrations of supernatant used were too low to produce a measurable glucose signal. Therefore, a further trial was conducted, incorporating heat as a mechanism of glycogen breakdown, in case the first trial failed due to poor enzymatic conversion. The solutions used in this trial are below:

1. 200 µl heated supernatant + 500 µl of 0.2 M sodium acetate buffer
2. 200 µl heated glycogen solution + 500 µl of 0.2 M sodium acetate buffer
3. 200 µl glycogen solution + 500 µl of 0.2 M sodium acetate buffer + 40 µl enzyme solution
4. 200 µl supernatant + 500 µl of 0.2 M sodium acetate buffer + 40 µl enzyme solution
5. 400 µl supernatant + 500 µl of 0.2 M sodium acetate buffer + 40 µl enzyme solution
6. 200 µl supernatant + 10 µl glucose solution
7. 400 µl supernatant + 10 µl glucose solution
8. 200 µl distilled water + 10 µl glucose solution
9. 400 µl distilled water + 10 µl glucose solution

For Solution 1, 0.5 ml of supernatant was placed in an Eppendorf tube and incubated in a water bath (50°C) overnight. For Solution 2, 0.0302 g of bovine glycogen (Sigma) was dissolved in distilled water to make a 0.01 g ml⁻¹ glycogen solution. Then, 0.5 ml of this glycogen solution was pipetted into an Eppendorf tube and left in a 50°C water bath overnight. This solution acted as a positive control for the effect of temperature on glycogen breakdown, while Solution 1 would determine if glycogen was present in sea cucumber tissue. Solutions 3-5 facilitated comparison of the enzymatic technique versus the heating conversion method, while Solutions 6-9 were positive controls to ensure the assay was working, and to determine baseline levels of glucose in supernatants.

All of solutions 1 – 9 were left overnight (for Solutions 1 and 2 the buffer was left to sit alone while the supernatant and glycogen solution were heated). The following morning, 10 µl of each solution was run through a glucose assay.

The results of this trial showed that heating 200 µl supernatant at 50°C overnight was suitable for glycogen breakdown, generated sufficient glucose to be detectable by the assay, and did not unduly contribute to baseline absorbance. This outcome was especially favourable as the amyloglucosidase enzyme was unable to be transported to Tonga. For the trials described above, the initial homogenisation step used a ratio of 0.55 g sea cucumber tissue per ml of

seawater. For each biochemical assay performed in Tonga, approximately 0.5 g of sea cucumber tissue was homogenised with 1 ml of seawater (the weight of each tissue taken was recorded).

2. 2. 5. 2. Tonga biochemistry methods

Biochemical analyses were only performed for the 10df and 10dl groups because of resource and time constraints. For both groups, coelomic fluid and body wall (0.5 ± 0.1 g) samples were taken from each animal and frozen. The coelomic fluid samples were taken by inserting a needle attached to a syringe into the body cavity through the body wall and drawing the fluid. Occasionally, the needle would block, and thus coelomic fluid was taken after an incision had been made with a scalpel through the body wall. The body wall samples were taken from the mid-ventral section of the body using a scalpel, and then frozen. For homogenisation, body wall samples were thawed and then chopped into pieces with a scalpel before being homogenised using a powered homogeniser on its highest setting for 80 seconds. The homogenate was refrigerated, centrifuged at 10 000 rpm for ~ 2 minutes until a pellet had formed and the supernatant taken and frozen until assayed. The coelomic fluid was used directly in the assay. All three substrates (glucose, glycogen and lactate) were measured in both the coelomic fluid and body wall.

Glucose and glycogen assays were carried out using the Sigma-Aldrich GAHK20 Glucose Assay Kit according to the manufacturer instructions. This assay works by enzymatically converting glucose to glucose-6-phosphate, which is then converted to 6-phosphogluconate, a step that also results in the formation of reduced nicotinamide-adenine dinucleotide (NADH). The increase in NADH corresponds to an increase in absorbance at 340 nm. To measure glycogen concentration, approximately 200 μ l of body wall supernatant and coelomic fluid samples were heated to 50 °C for 12 hours. Ten μ l of these heated samples were run through a glucose assay to measure glycogen as glucose (Sigma-Aldrich GAHK20).

Lactate assays were carried out using the Megazyme L-lactic acid assay kit according to manufacturer instructions. To quantify lactate acid two reactions are required; the first where lactate is oxidised to pyruvate, forming NADH. To prevent the reverse reaction, the pyruvate is then rapidly converted, thus maintaining NADH in solution, where its absorbance at 340 nm is proportional to the lactate present in the sample.

2. 2. 5. 3. Lactate

The absorbance results from the assay procedure were converted into lactate concentration (g l^{-1}) using Equation 2.7 (Megazyme L-lactic acid assay kit):

Equation 2.7

$$((V \times MW) \div (e \times d \times v)) \times \Delta A_{L-lactate}$$

Where V = final volume (ml); MW = 90.1 (molecular weight of lactate); e = 6300 (extinction coefficient); d = light path (1 cm) of the cuvette; v = sample volume (ml); $\Delta A_{L-lactate}$ = (sample absorbance - blank absorbance). To calculate the content of lactate (mg g^{-1}), the concentration of lactate (g l^{-1}) was divided by the body wall sample weight (g).

2. 2. 5. 4. Glucose/Glycogen

Equations 2.8 and 2.9 specified in the Sigma HK Glucose Assay Kit Procedure Bulletin were used to convert absorbance values to glucose concentration:

Equation 2.8

$$A_{Total\ Blank} = A_{Sample\ Blank} + A_{Reagent\ Blank}$$

Equation 2.9

$$mg\ glucose\ ml^{-1} = (\Delta A)(TV)(Glucose\ Molecular\ Weight)(F) \div (e)(d)(SV)(Conversion\ Factor\ for\ \mu g\ to\ mg)$$

Where $\Delta A = A_{Test} - A_{Total\ Blank}$, TV = Total Assay Volume (1.01 ml), SV = Sample Volume (0.01 ml), $Glucose\ MW = 1802\ \mu g\ \mu moles^{-1}$, F = Dilution Factor from Sample Preparation, e = Extinction Coefficient for NADH at 340 nm (6.22), d = Light path (cm), Conversion Factor for μg to mg (1000). Glucose levels were then expressed as mg ml^{-1} (for coelomic fluid) and ug g^{-1} (for body wall). To calculate sample glycogen concentration, the glucose concentration of the unheated sample was subtracted from the total glucose sample (the heated sample). The units of glycogen were expressed as units of glucose in ug g^{-1} for the body wall.

2. 2. 6. Determination of regeneration

Following the measurement of physiological end-points and the collection of coelomic fluid and body wall samples for biochemical analysis, the state of internal organs was determined. In addition, observations were made as to whether there was any evidence of feeding (sediment in the intestines).

To determine if there was a difference in organ presence/absence between treatments, the number of whole animals with each organ (respiratory tree, intestine or gonad) or combination of organs (respiratory tree and intestine – represented in **Figure 2.4**, **Figure 2.5**, & **Figure 2.6**, as RT and I) was divided by the total number of whole animals within that treatment within each group (6dl, 10dl, and 10df). For the category RT and I, the data are also represented in the intestine part (< ½ regenerated), intestine intact (connected to both anus and mouth), and/or respiratory tree categories to indicate the percent of animals with each of these organs with respect to other treatments. So respiratory tree and intestine part/intestine full categories represent animals which only had a respiratory tree or an intestine but can also include those which had both. The stage of respiratory tree regeneration was not noted, so respiratory tree is only presence/absence data but it should be noted that it is possible that there were different levels of regeneration for this organ.

2. 2. 7. Data analysis

All data were analysed to test for differences between treatments and between locations (laboratory and field). In the laboratory experiment, the assumption was made that there was no significant difference between bags and tanks in terms of their conditions. This was because each treatment was set-up twice – once in each tank, and bags were randomised to prevent experimental bias in the form of stratification. Furthermore, the bags were all cut from the same shade cloth material and to the same size, and thus had the same volume. They were also kept in the same depth of water in each tank, and both tanks had the same speed water flow. Thus light, depth, and water flow conditions did not differ between bags between treatments thus negating the need to account for a bag or tank effect. It was also assumed that there was no significant difference in density between laboratory and field groups because of the confounding effect of budding. All physiological data was analysed for differences between budded and whole animals before analysing for treatment effects. To determine if there was a treatment effect on budding frequency, a test for independence was conducted.

During the course of the experiments, some animals budded. Budded animals themselves could be animals formed from the anterior of the original animal or the posterior; referred to as anterior and posterior buds, respectively. It was not known if budding and budding location would significantly impact the endpoints measured. To test this all animals (whole animals, anterior and posterior buds) were initially treated as identical. For every treatment, and end-point (buds were only utilised in MO₂ and AER experiments), an overall mean value

was calculated (i.e. a mean was calculated for 6dl MO₂, 6dl AER; 10 dl MO₂, etc.). Each individual rate was then expressed as a percentage of this overall mean. The percentage values of the three groups (whole animals, anterior and posterior buds) was then statistically tested (parametric ANOVA for MO₂; Kruskal-Wallis ANOVA for AER) to determine whether these percentage values statistically differed.

Two outlier exclusion methods; Chauvenet's criterion (Sabade & Walker, 2002) and rejecting data points which lay beyond two standard deviations from the mean, were compared on an initial data-set. The two methods yielded very similar results, except the Chauvenet's criterion allowed slightly more robust outlier identification. Therefore, this technique was applied to all of the data-sets throughout this study for consistency.

For all data-sets, the Shapiro-Wilk test and Bartlett's tests were used to test for normality and homogeneity of variances respectively. If the data were not normally distributed they were transformed and re-tested for normality, with the Fligner-Killeen test used on transformed data for assessment of equal variances. Data that passed tests were assessed using parametric statistics, otherwise non-parametric tests were performed.

Using the statistical software Rstudio, Analysis of Variance (ANOVA), followed by a post-hoc Tukey HSD test, was applied to normal homogenous data. When the data were not normal, the non-parametric Kruskal-Wallis test with a post-hoc Tukey and Kramer (Nemenyi) test were used. An alpha level of 0.05 was used for all tests. A Chi-square test was used to determine if there was a treatment effect on asexual reproduction. This test was used this to test the observed outcome against an *a priori* expected outcome.

When determining if there was an effect of treatment in the field group alone (which had only two treatment groups) a two-sample t-test was used. When comparing between treatments within the 10dl or 6dl groups, which each had three treatment groups, a one-way ANOVA was used (or a Kruskal-Wallis test if the data was non-parametric). To compare between laboratory and field groups, because there was no sham group in the field, the sham treatment in the laboratory group was excluded from the analysis. A two-way ANOVA was used to investigate if there was an effect of treatment or location, as well as an interaction between treatment and location on the end-point measured. When the data was non-parametric, Kruskal-Wallis tests were used to determine if there was a significant effect of treatment or location on the end-point, but not the interaction between these two factors, because it is outside the scope of that statistical test.

Table 2.1. Summary of statistical methods

End-point analysed	Analyses performed
MO ₂	One-way ANOVA, post-hoc Tukey HSD test, two-way ANOVA
AER	Kruskal-Wallis test
Glucose body wall	two sample t-test, one-way ANOVA, Kruskal -Wallis test
Glycogen body wall	two-way ANOVA, post-hoc Tukey HSD test, two sample t-test, one-way ANOVA
Budding	Chi-square test for independence

2. 3. Results

Overall, there was a treatment effect observed in the 10df group but not in either of the laboratory groups. Internal organ regeneration was evident across all three treatment groups, as were the products of asexual reproduction. The frequency of budding was highest in the laboratory study compared with the field group.

2. 3. 1. Internal Organ Regeneration

The intestine and respiratory tree were always the first two organs to regenerate, with there being some variation between which of these two organs regenerated first. In the 10df group only one animal (a control whole animal) had sediment in the intestines, but no sediment was found in the intestines of any animals from the 10dl and 6dl groups. In the 10df and 10dl groups no animals were found with gonads, or any form of gonad regeneration to the naked eye.

For the 6dl group, all of the control animals had a respiratory tree, while 75% had an intact intestine (**Figure 2.4**). In the sham treatment, 100% of animals had a respiratory tree and only 25% had an intact intestine (**Figure 2.4**). In the excised treatment 67% of sea cucumbers had a respiratory tree and 50% had an intestine partially regenerated. There were no animals in this treatment that had a completely intact intestine.

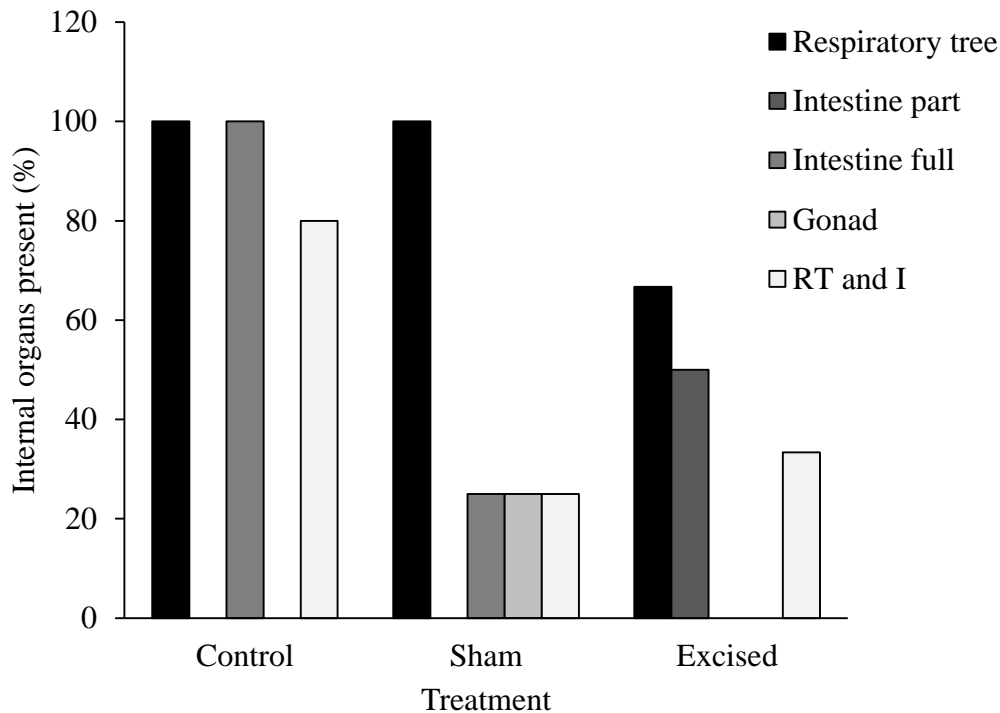


Figure 2.4. The percentage (%) total of 6dl whole animals with internal organs per experimental treatment. Each bar represents the percentage of animals with each individual organ, some had both a respiratory tree and an intestine (RT and I), some had the intestine partially regenerated (Intestine part) while others had an intestine connected from head to anus (Intestine full); control (n = 8), sham (n = 7) and excised (n = 8).

The results for the 10dl group show that in the control group, 100% of animals had a respiratory tree, 17% had a partially regenerated intestine, 83% had a fully intact intestine, and 17% had gonads (**Figure 2.5**). In the sham group, the majority of animals had a respiratory tree and just over half had an intact intestine. Partial intestinal regeneration was evident (29% of animals), and approximately a quarter of animals had gonads. In the excised group, most animals had respiratory trees (75%) and a few had a partially regenerated intestine (25%). No animals in the excised group had an intact (fully regenerated) intestine and none had both a respiratory tree and intestine. Therefore, 10 days after the beginning of the laboratory experiment, it appeared that all three treatment groups had some animals regenerating their intestines.

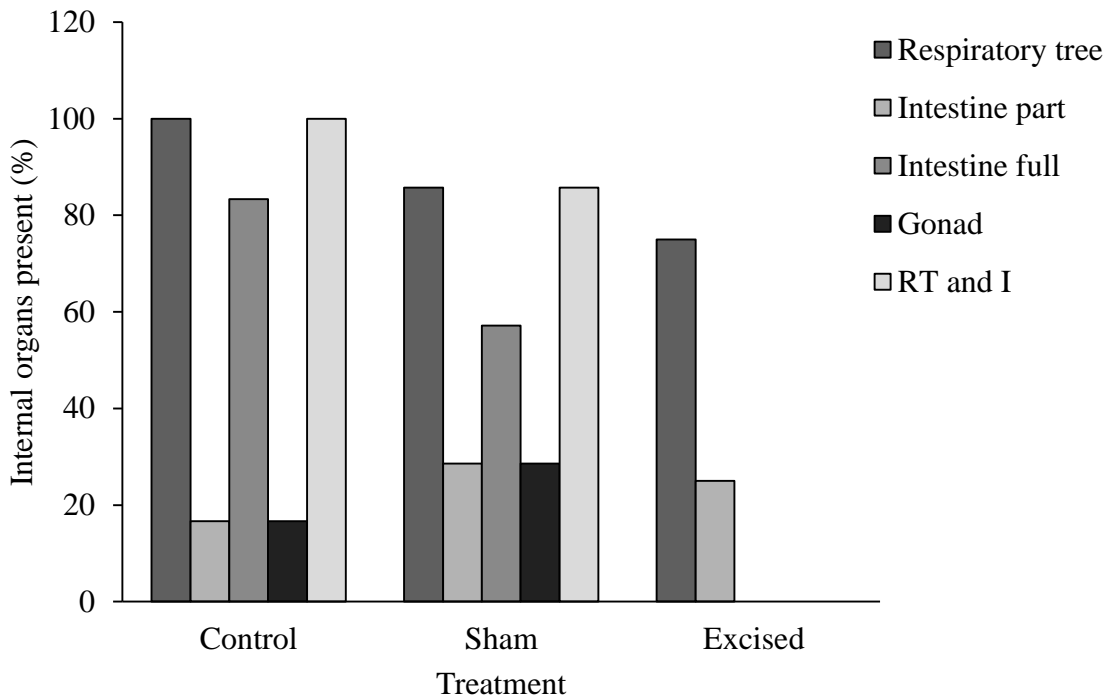


Figure 2.5. The total percentage of 10dl whole animals with internal organs per treatment. Each bar represents the percentage of animals with each individual organ, some had both a respiratory tree and an intestine (RT and I), some had the intestine partially regenerated (Intestine part) while others had an intestine connected from head to anus (Intestine full); control (n = 10), sham (n = 8) and excised (n = 11).

In the 10df group, one quarter of the total excised sea cucumbers in this group had a partially regenerating intestine, while a further 25% had a fully regenerated intestine (**Figure 2.6**). Additionally, all excised animals had regenerated a respiratory tree, and some animals had both a respiratory tree and an intestine. All of the respiratory trees in the 10df group (whole animals) were very small and only in the early stages of regeneration. In the case of the controls, all animals had respiratory trees and complete intestines indicating that none of the animals eviscerated during the course of the experiment and/or they had intact intestines before the experiment began. No animals in either the control or the excised treatment groups had gonads.

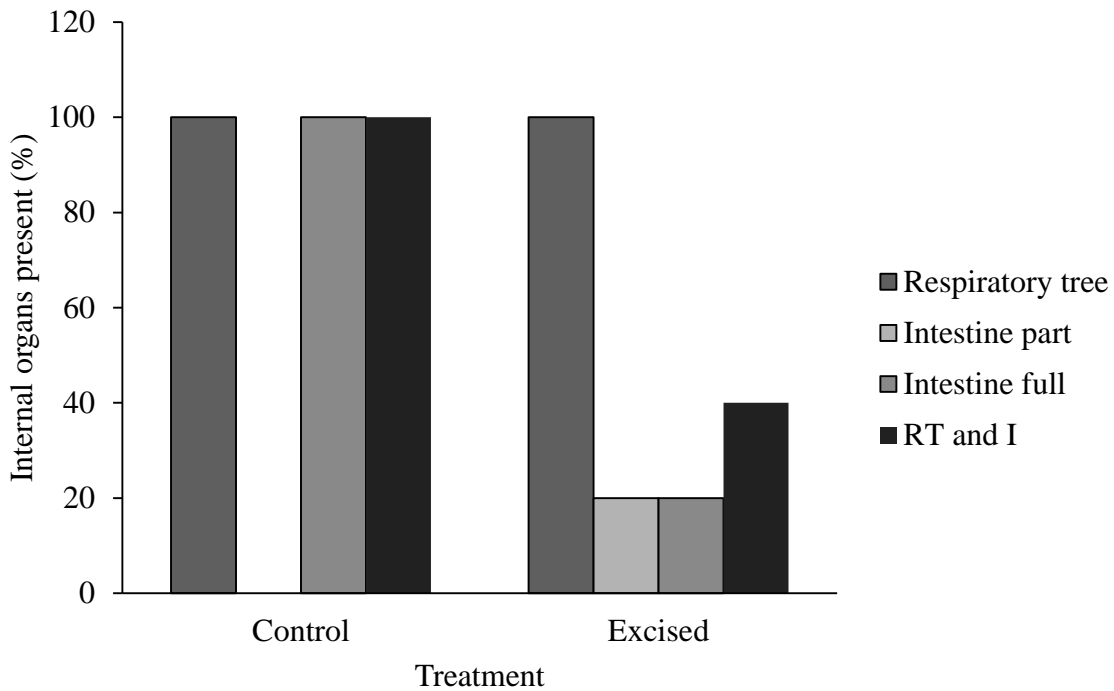


Figure 2.6. The total percentage of 10df whole animals with internal organs per treatment. Each bar represents the percentage of animals with each individual organ. Some had both a respiratory tree and an intestine (RT and I), some had the intestine partially regenerated (Intestine part) while others had an intestine connected from head to anus (Intestine full) - control (n = 8) and excised (n = 11).

2. 3. 2. Asexual reproduction

A chi-square test on excised and control, field and laboratory groups (sham excluded) suggested that any differences were independent of treatment (control or excised) and location (laboratory or field) ($\chi^2 = 1.712$, $p = 0.194$). However, the excision and cutting may potentially act as a trigger for fission given the differences observed in the counts (**Figure 2.7**).

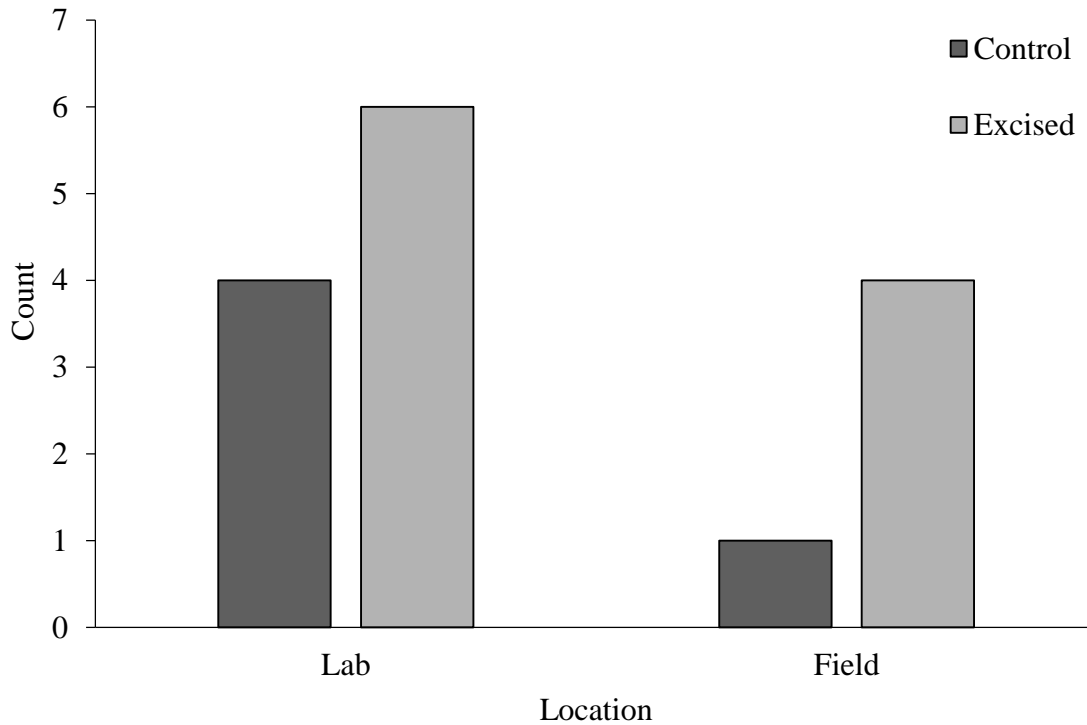


Figure 2.7. Count data displaying the number of whole animals which budded in the 10dl and 10df groups (grouped as Laboratory and Field here) as a function of treatment within location.

2. 3. 3. Physiology

2. 3. 3. 1. MO_2

2. 3. 3. 1. 1. *Budded animals MO_2*

The overall MO_2 was calculated for each animal as a percent of each group treatment mean (each group being either the 6dl, 10dl or 10df group) (**Figure 2.8**). This was used as a tool to determine if there was a significant difference in MO_2 between animal types (whole animals, anterior and posterior buds). Results of a one-way ANOVA on square root transformed data followed by a post-hoc Tukey test revealed that whole animals had a significantly higher MO_2 than both anterior buds ($p < 0.001$) and posterior buds ($p < 0.001$). There was no significant difference in MO_2 between bud types ($p = 0.954$). Based on the statistical analysis of the data in **Figure 2.8**, buds and whole animals were not able to be grouped and were thus analysed separately.

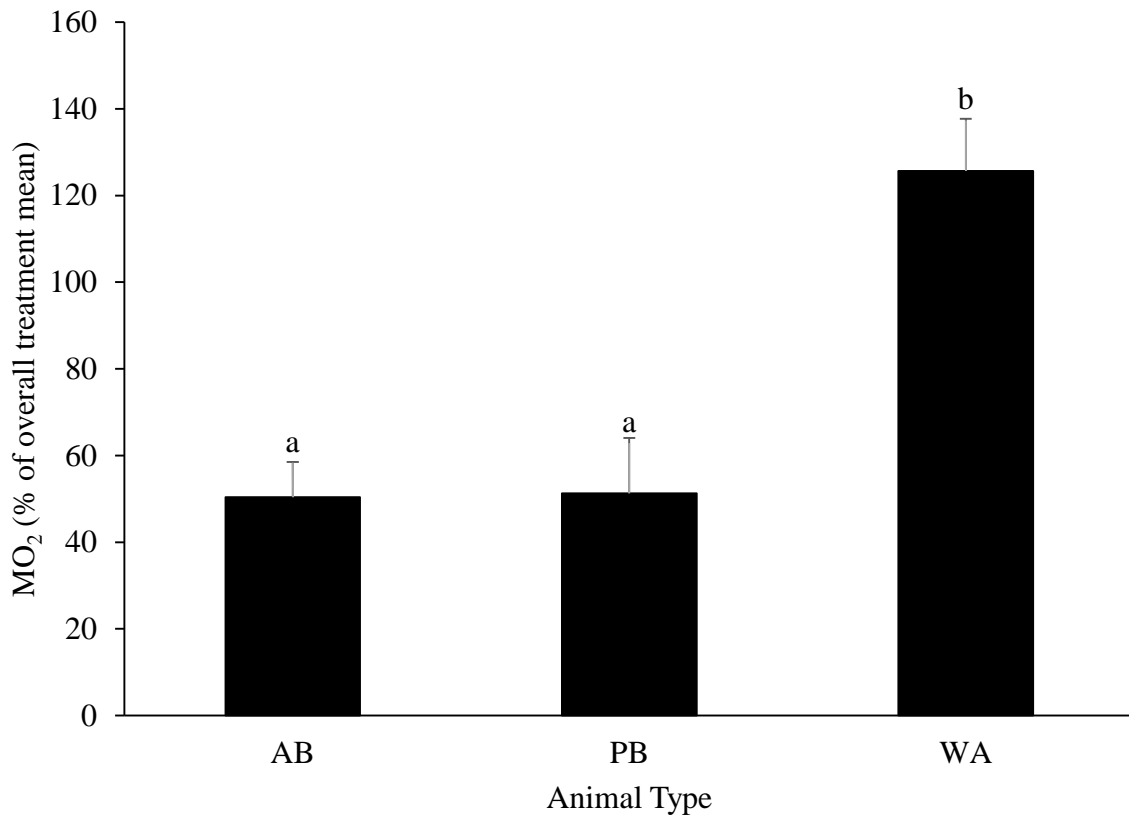


Figure 2.8. Bars displaying MO₂ (% of overall treatment mean + standard error) for posterior buds (PB; n = 15), anterior buds (AB; n = 14) and whole animals (WA; n = 40). Bars sharing letters are not statistically significantly different as determined by a one-way ANOVA followed by a Tukey post-hoc test. Data was square root transformed for analysis. There was a significant difference between animal types (Fstat = 13.35, df = 2, 66, p < 0.001).

2. 3. 3. 1. 2. 10dl group bud analysis

The 10dl buds were the only budded group with a sample size large enough (n > 4 buds per treatment) to analyse for an effect of treatment on MO₂. There was no significant difference in MO₂ between treatments (Fstat = 1.063, df = 2, 13, p = 0.374) as shown by a one-way ANOVA on untransformed data (**Figure 2.9**).

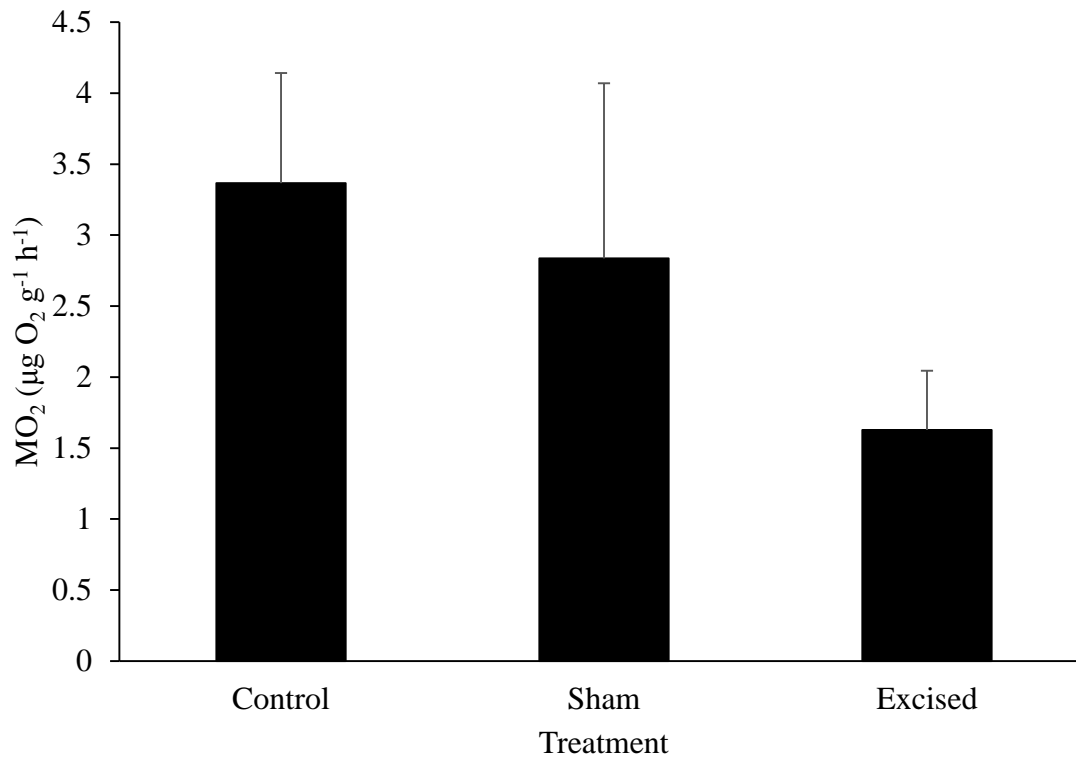


Figure 2.9. Mean MO₂ (µg O₂ g⁻¹ h⁻¹) + standard error for each treatment; control (n = 6), sham (n = 5), excised (n = 5), in the 10dl bud group.

2. 3. 3. 1. 3. Whole animals: Overall MO₂

The results of a two-way ANOVA on square root transformed data for the 6dl vs. 10dl (whole animals, i.e. no buds) showed that there was no significant effect of treatment (Fstat = 0.89, df = 2, 24, p = 0.424) or interaction between treatment and time (Fstat = 1.15, df = 2, 4, p = 0.333) on MO₂ (**Figure 2.10**). However, the MO₂ of the 10dl group was significantly higher than that of the 6dl group (Fstat = 9.41, df = 1, 24, p = 0.005) (**Figure 2.10**).

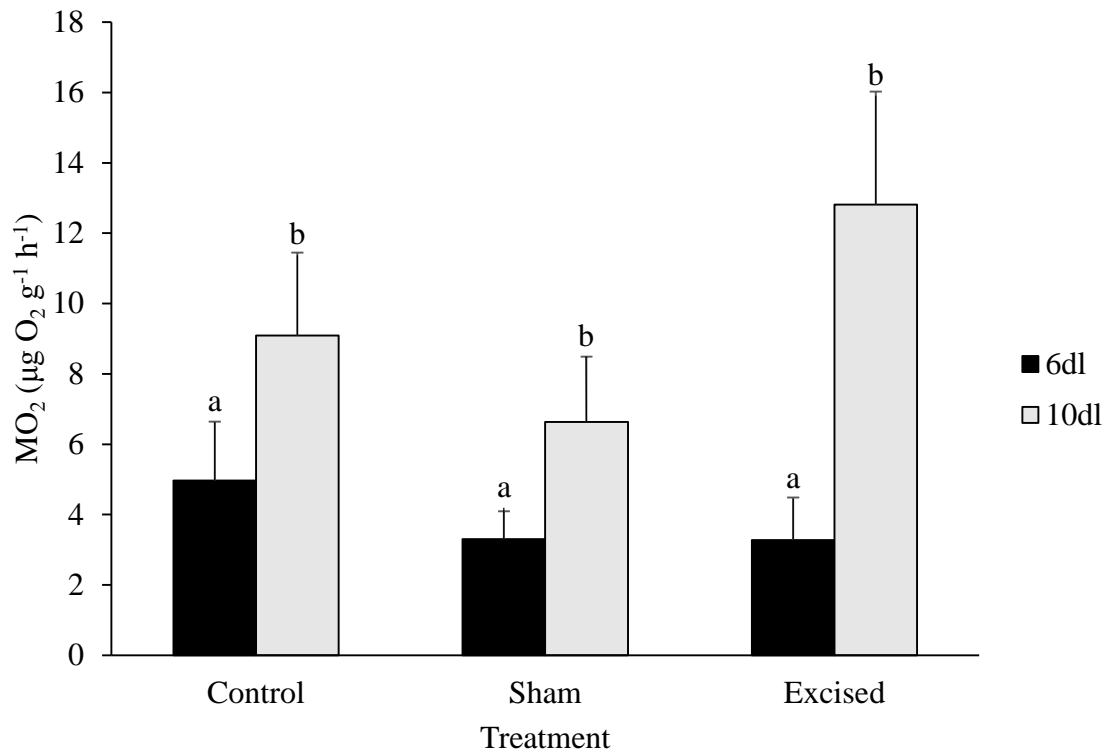


Figure 2.10. Mean MO_2 ($\mu\text{g O}_2 \text{g}^{-1} \text{h}^{-1}$) + standard error for the 6dl and 10dl groups per treatment ($n = 4-7$). Bars sharing letters are not statistically significantly different from one another as determined by a two-way ANOVA.

A two-way ANOVA on square root transformed data for 10dl vs 10df groups using only the control and excised treatment groups (whole animals) showed that there was no significant effect of treatment on MO_2 ($F_{\text{stat}} = 0.596$, $df = 1, 16$, $p = 0.451$) (**Figure 2.11**). There was a significant effect of location ($F_{\text{stat}} = 9.01$, $df = 1, 16$, $p = 0.009$) and interaction between treatment and location ($F_{\text{stat}} = 6.04$, $df = 1, 16$, $p = 0.026$) on MO_2 . A Tukey post-hoc test showed that laboratory control group had a significantly higher MO_2 than the field excised group ($p = 0.040$) and the laboratory excised group had a significantly higher MO_2 than the excised field group ($p = 0.008$), the controls were not significantly different between locations ($p = 0.866$). A two sample t-test on the 10df group alone showed that the control animals had significantly higher MO_2 than the excised sea cucumbers ($t_{\text{Stat}} = 3.67$, $df = 8$, $p = 0.006$). A one-way ANOVA on the 10dl group alone showed that there was no significant difference in MO_2 between treatments ($F_{\text{stat}} = 1.563$, $df = 2, 14$, $p = 0.244$).

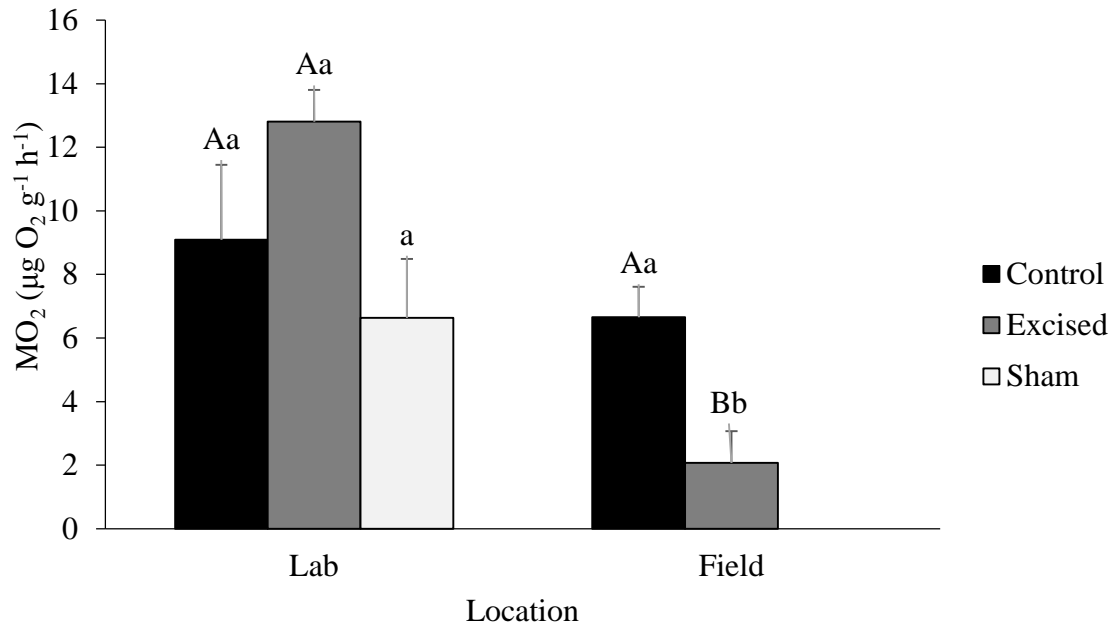


Figure 2.11. MO₂ (µg O₂ g⁻¹ h⁻¹) + standard error for the 10dl and 10df groups per treatment (n = 4-7). Bars sharing lowercase letters within a location are not significantly different as determined by a two-way ANOVA, a two sample t-test and a post-hoc Tukey test. Bars sharing uppercase letters between locations but within treatments are not statistically significantly different from each other.

A linear regression showed a significant positive relationship between mass and MO₂ in all three groups (6dl, 10dl, and 10df) (**Figure 2.12**).

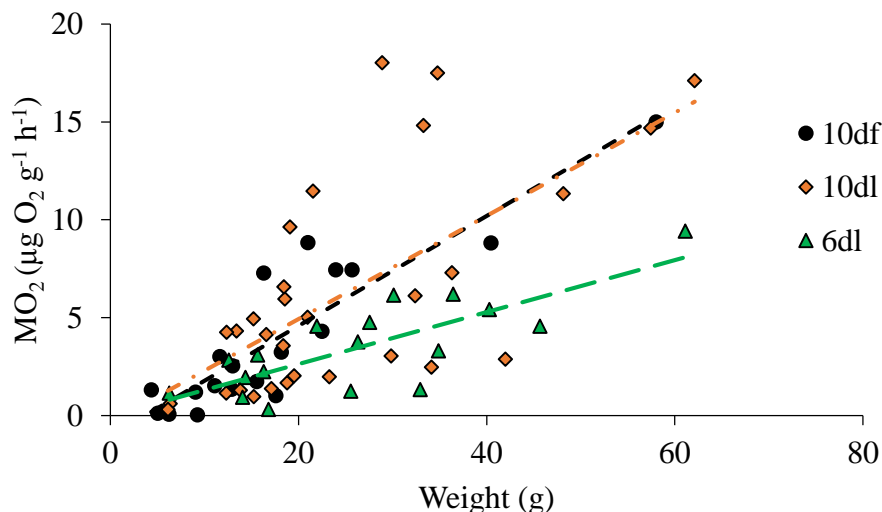


Figure 2.12. Scatterplot displaying MO₂ as a function of animal wet weight (g) for animals in the 6dl, 10dl, and 10df group. Linear regression (6dl: MO₂ = 0.13x - 0.01, R² = 0.604, p < 0.001; 10dl: MO₂ = 0.26x - 0.34, R² = 0.44, p < 0.001; 10df: MO₂ = 0.281X - 1.033, R² = 0.81, p = < 0.001).

2. 3. 3. 2. Ammonia Excretion Rate

As for MO_2 , the percentage overall AER per treatment per group was calculated to assess if there was a significant difference in AER between animal types (anterior buds, posterior buds, and whole animals) (**Figure 2.13**). A Kruskal-Wallis test showed no statistically significant difference in the mean ranks of AER (% of the mean treatment values) between animal types ($H = 2.686$, $df = 2$, $p = 0.261$). Therefore, the animal types within each treatment were grouped together for the proceeding data analysis.

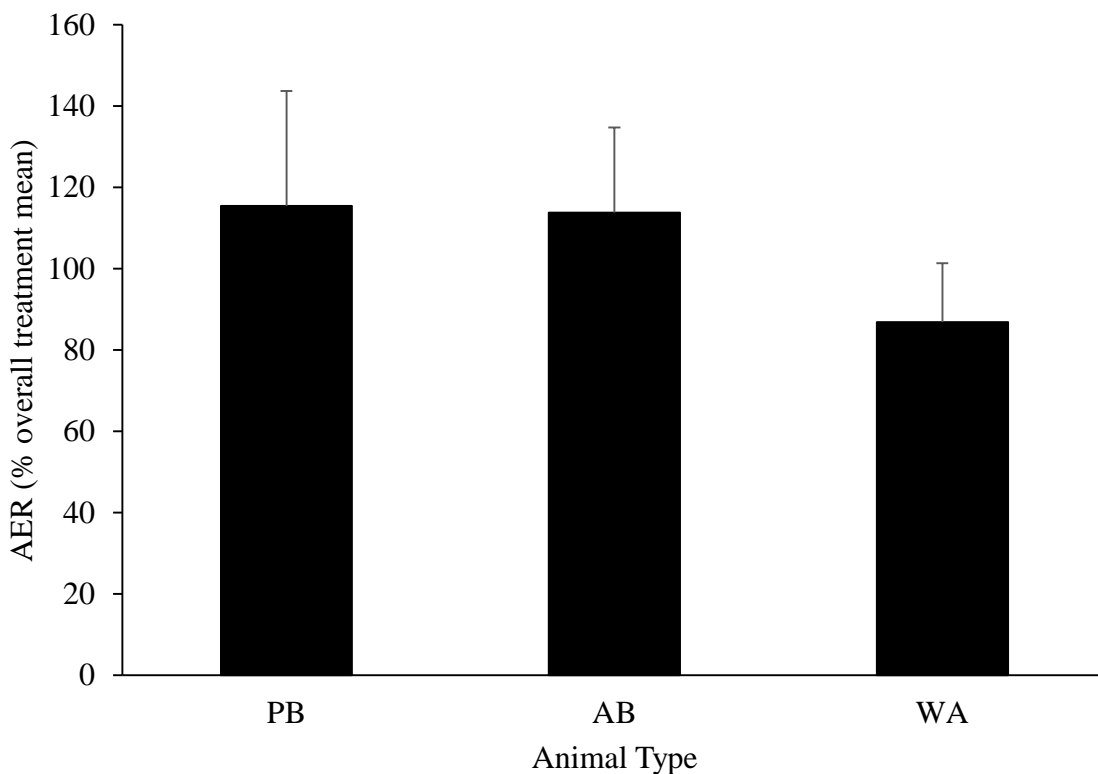


Figure 2.13. AER (% of the treatment mean + standard error), for different animal types where PB; posterior bud ($n = 15$), AB; anterior bud ($n = 15$) and WA; whole animal ($n = 44$).

2. 3. 3. 2. 1. Comparison of whole animals vs buds - AER

To determine if there was a significant effect of treatment or time in the 6dl and 10dl groups, a Kruskal-Wallis test was performed. There was no significant effect of time ($H = 0.037$, $df = 1$, $p = 0.848$) or treatment ($H = 1.198$, $df = 2$, $p = 0.549$) on mean ranks of AER ($\mu\text{g g}^{-1} \text{h}^{-1}$) (**Figure 2.14**).

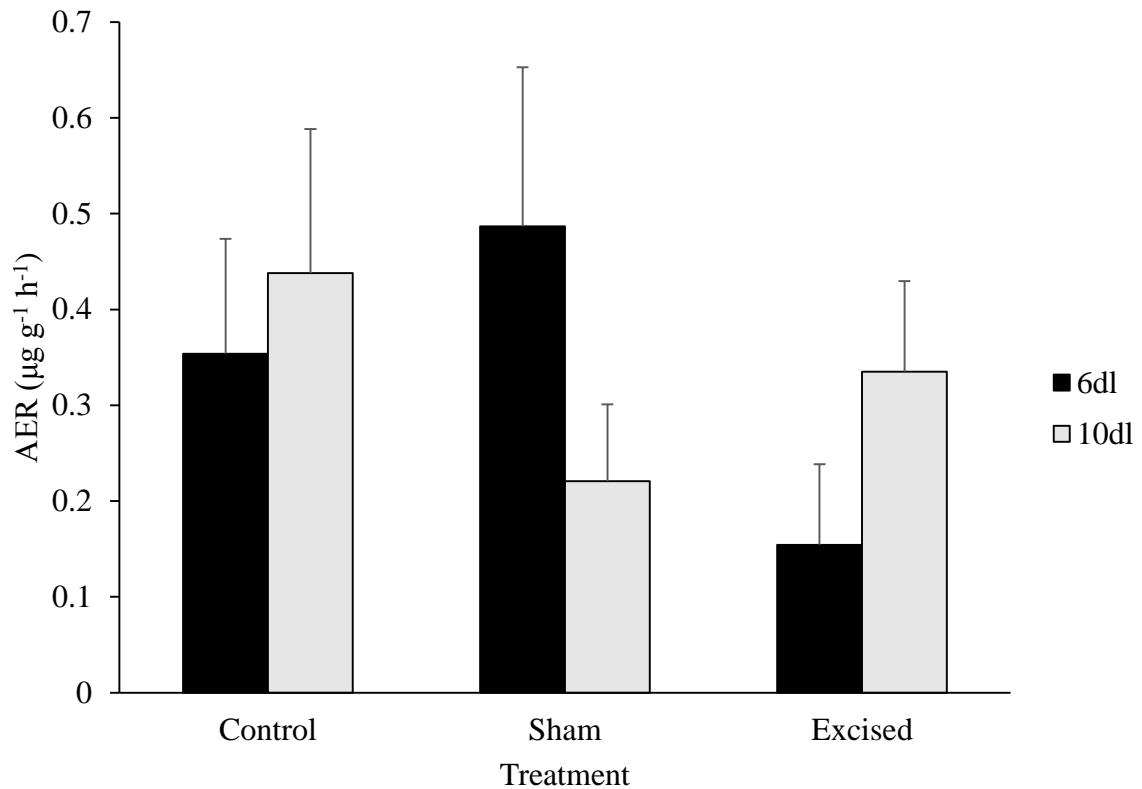


Figure 2.14. Mean AER ($\mu\text{g g}^{-1} \text{h}^{-1}$) + standard errors for treatments within each of the 6dl (control; $n = 8$, sham; $n = 7$, excised; $n = 7$) and 10dl (control, $n = 11$; sham, $n = 11$; excised, $n = 11$) groups for each treatment. Data presented here are pooled for buds and whole animals.

2. 3. 3. 2. 2. Comparison between 10dl and 10df groups - AER

A Kruskal-Wallis test showed that there was no significant difference in the mean AER ($\mu\text{g g}^{-1} \text{h}^{-1}$) ranks between treatments (sham excluded from laboratory group) ($H = 0.145$, $df = 1$, $p = 0.704$), but that there was a significant difference in the mean AER ($\mu\text{g g}^{-1} \text{h}^{-1}$) ranks between locations ($H = 5.64$, $df = 1$, $p = 0.018$) (**Figure 2.15**).

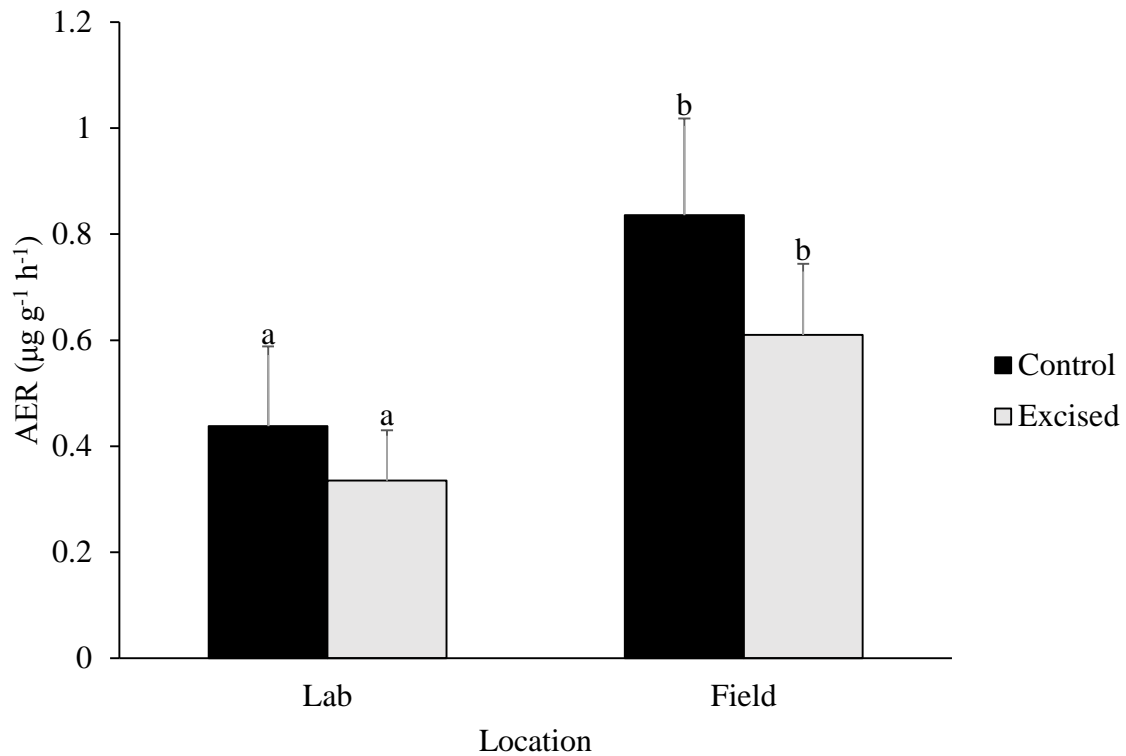


Figure 2.15. AER ($\mu\text{g g}^{-1} \text{h}^{-1}$) + standard errors for the 10dl (Laboratory groups: control, $n = 11$; excised, $n = 11$) and 10df (Field groups: control, $n = 8$; excised, $n = 11$) groups. Groups sharing letters are not statistically significant as determined by a Kruskal-Wallis test.

2. 3. 4. Biochemistry

Only coelomic fluid and body wall samples from whole animals in the 10dl and 10df groups were chosen for biochemical analysis due to logistical, resource and time constraints.

Therefore, in the following analysis there is no comparison between buds and whole animals because samples from budded animals were not assayed.

2. 3. 4. 1. Lactate

For both the laboratory and field studies the lowest value of lactate in the body wall was $0.00 \text{ mg lactate g}^{-1}$ ($n = 10$), and for the coelomic fluid was 0.00 g l^{-1} ($n = 14$). The highest lactate value for the body wall was 0.03 mg g^{-1} and for the coelomic fluid was 0.08 g l^{-1} . The assay limit of quantification was calculated to be $0.06 \text{ mg lactate g}^{-1}$ for body wall samples, and 0.03 g l^{-1} for coelomic fluid. None of the body wall lactate concentrations, and less than 5% of coelomic fluid values, met this criterion. Thus it was concluded that lactate production in sea cucumbers was low, irrespective of treatment in the current study, and no statistical analysis on these data was conducted.

2. 3. 4. 2. Glucose

A maximum coelomic fluid glucose concentration of 0.05 mg glucose ml⁻¹ and a minimum of 0.00 mg glucose ml⁻¹ was obtained during the course of this study. A total of 6 animals across both the 10df and 10dl data (treatments combined) had zero units of glucose in the coelomic fluid. Again, these levels were calculated as being below the limits of quantification, and thus the data were not analysed.

For body wall glucose concentrations, as determined by two-sample t-tests, a one-way ANOVA and Kruskal-Wallis tests, there was no significant effect of treatment on glucose concentration in the 10df group (tStat = -1.26, df = 8, p = 0.242) or the 10dl group (Fstat = 1.03, df = 2, p = 0.387), and no significant difference in glucose concentration between the laboratory or field groups (H = 0.952, df = 1, p = 0.329) (**Figure 2.16**).

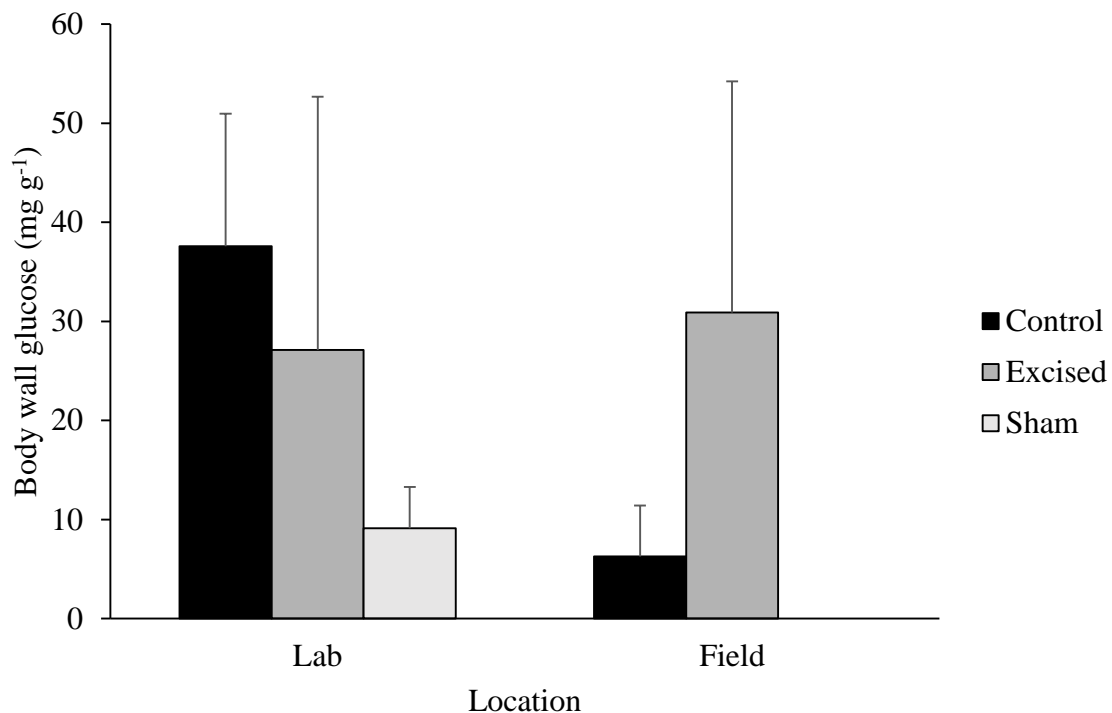


Figure 2.16. Mean body wall glucose concentration (mg g⁻¹) + standard errors for each treatment in each of the field (10df: control, n = 6; excised, n = 4) and laboratory (10dl: control, n = 5; sham, n = 6; excised, n = 4) experiments.

2. 3. 4. 3. Glycogen

A two-way ANOVA on square root transformed data for control and excised treatments from the 10df and 10dl groups showed that there was no significant effect of treatment on glycogen body wall concentration (Fstat = 1.25, df = 1, p = 0.283) (**Figure 2.17**). However,

there was a significant effect of location ($F_{\text{stat}} = 6.24$, $df = 1$, $p = 0.026$) and interaction between treatment and location ($F_{\text{stat}} = 6.67$, $df = 1$, $p = 0.022$). A post-hoc Tukey test revealed that there was a significantly higher body wall glycogen concentration in the excised field group than the excised laboratory group ($p = 0.014$), and there was almost a significant difference in glycogen body wall concentration between the treatment groups in the field ($p = 0.067$). It should be noted here that when a two-sample t-test was conducted on the field data alone, the result for the difference between treatments in glycogen was statistically significant ($t_{\text{Stat}} = -2.53$, $df = 8$, $p = 0.035$). To determine if there was a significant difference between all three treatments in the laboratory (because sham treatment group was excluded in the above analysis for location) a one-way ANOVA on the 10dl group alone was conducted and showed that there was no significant difference in glycogen concentration between treatments ($F_{\text{stat}} = 0.91$, $df = 2$, $p = 0.435$).

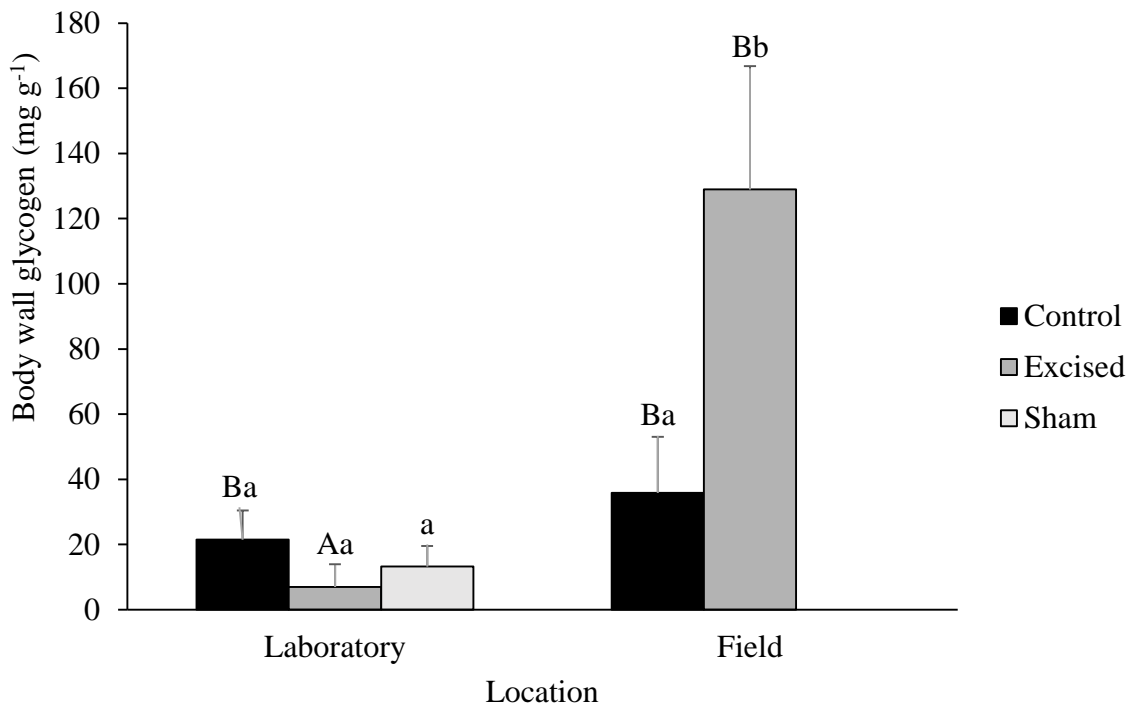


Figure 2.17. Mean glycogen body wall concentration (mg g^{-1}) + standard errors for all treatments grouped by location where laboratory refers to 10dl group (control, $n = 4$; excised, $n = 4$; sham, $n = 5$) and where field is 10df group (control, $n = 6$; excised, $n = 4$). Bars sharing lowercase letters indicate that treatments within a location are not statistically significantly different, while bars sharing uppercase letters indicate that treatments between locations are not statistically significantly different as determined by a two-way ANOVA on square root transformed data and two-sample t-tests.

2.3.5. O:N

The average O:N for the 6dl groups across all treatments ranged between 13 and 25, while the values ranged between 15 and 32 in the 10dl group (Figure 2.18). In the 10df group, the average O:N ranged between 4 and 14 (Figure 2.18). There appeared to be differences in the O:N between groups and between treatments within groups, however, this was not statistically analysed due to the approach of calculating group, rather than individual, means (Figure 2.18).

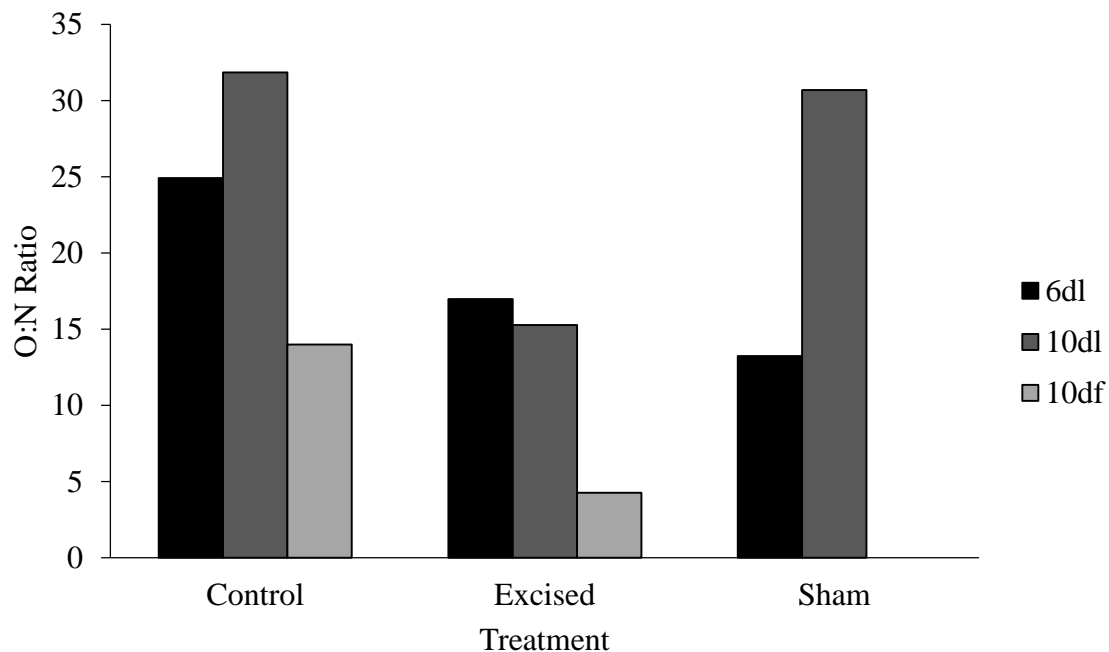


Figure 2.18. Average O:N for whole animals per treatment for each experimental group. Plotted values represent group means for; 10dl (control, n = 2; sham, n = 5; excised, n = 2), 6dl (control, n = 3; sham, n = 4; excised, n = 2), 10df (control, n = 6; excised, n = 3).

2.4. Discussion

2.4.1. Internal organ regeneration

The intestinal growth of sea cucumbers that had undergone evisceration in this study was similar to that following evisceration in the sea cucumber species *Holothuria scabra* (Bai, 1994) and *Thyone briareus* (Kille, 1935). In the current study, 53% of whole excised animals displayed intestinal growth reaching approximately 1/3rd of the way down the length of the animal after 6 days. Both *H. scabra* and *T. briareus* demonstrated regenerating intestinal tissue by days 6 and 5 respectively (Kille, 1935; Bai, 1994). As would be expected, excised

animals were the only treatment with regenerating intestines. However, only a quarter of the sham animals had a full intestine with the rest displaying no intestine and no intestinal regeneration. This indicates that the sham animals either; had no intestines before the study began and had not regenerated intestines by 6 days, or that they eviscerated their intestines over the course of the experiment and had not begun regenerating them. Given that *S. horrens* can eviscerate through a body wall rupture (Dolmatov et al., 2012), I suspect that the sham animals eviscerated after they were put into the experiment. This is because, if the sham animals had no internal organs before they went into the experiment, some sort of intestinal regeneration would be expected, as was shown in the excised animals. This was not observed. However, if they did not eviscerate straight away, but one or two days after they went into the experiment, it may explain why there is no intestinal growth or visible regeneration by Day 6.

The sea cucumber species *Holothuria glaberrima* and *Apostichopus japonicus* can regenerate a fully functional intestine in approximately three weeks (García-Arrarás et al., 1998; Shukalyuk & Dolmatov, 2001), whereas *H. scabra* can achieve the same in only seven days (Bai 1971 in García-Arrarás & Greenberg, 2001). This demonstrates considerable variation in intestinal regeneration rates between sea cucumber species. The preliminary regeneration investigation carried out in the present study prior to the commencement of the actual regeneration experiment, indicated that *S. horrens* could regenerate a fully functional intestine in 10-days. However, this study demonstrated intra-specific differences in regeneration rates.

None of the sea cucumbers in the 10dl and 6dl groups had sediment in the intestines indicating that they were unlikely to be feeding during the experiment. Food was available to the experimental animals, but the lack of sediment in the intestines could also have been due to the nocturnal feeding behaviour of the animals which means they generally have empty intestines during the day (Eriksson et al., 2007). Of the whole excised animals in the 10dl study, only 25% showed partial intestine regeneration and no animals had fully regenerated an intestine. This contrasts to the 10df group where 50% of excised animals had begun to regenerate an intestine, and one animal had even regenerated a fully intact intestine. This indicates that 10 days was too short a time for complete intestinal regeneration for all individuals, and that there is inter-individual variation in intestinal regeneration times. A similar regeneration study conducted in the Kingdom of Tonga on *S. horrens* indicated that it takes 30-days for internal organ regeneration to complete (Barker, 2007). The difference between individuals may be due to a variety of factors including, but not limited to; the age of

individual animals, the feeding behaviour of each animal leading up to excision (starvation in the days leading up to autotomy can aid in the regenerative process) or the number of times that the animal has lost its internal organs or been wounded previously (Korschelt, 1927; Needham, 1952). The discrepancy in intestinal regeneration times between the laboratory and field is likely a reflection of laboratory conditions having an effect on regeneration time (García-Arrarás & Greenberg, 2001).

The same variables that affect everyday survival and growth of an organism affects its regeneration (Morgan, 1901). It is generally accepted that temperature affects regeneration, with higher temperatures (up to a certain point) generally resulting in elevated regeneration rates, and this has been demonstrated in the brittlestar species *Ophionotus victoriae* (Morgan, 1901; Clark et al., 2007). Unfortunately, it cannot be inferred whether this may have had an effect in the current study as variations in temperature between the laboratory and field studies were not monitored.

Salinity, oxygen and food availability are also known to influence regeneration rates in echinoderms (Morgan, 1901). To the best of my knowledge, there are no studies on the effects of any of these variables on regeneration specifically in sea cucumbers. However, in the brittlestar, *Ophiophragmus filograneus*, a lower salinity resulted in decreased regeneration relative to higher salinities (Talbot & Lawrence, 2002). This would be an interesting parameter to investigate in future studies of regeneration in holothurians.

In starved animals, regeneration time is often decreased until nutrients become available to fuel the process (Fielman et al., 1991). The sea cucumber *Chiridota rigida* has been shown to transport free amino acids from the external medium into the internal cavity of the animal, where they are either catabolised, synthesised into proteins or remain free amino acids (Ahearn & Townsley, 1975). The ability to absorb amino acids dissolved in seawater has been demonstrated in other sea cucumber species such as *Thyone briareus*, *Leptosynapta inhaerens* and *Parastichopus californicus* (Stephens & Schinske, 1961; Brothers et al., 2015). It is possible that there was a higher availability of free amino acids in the field environment which may be contributing to the differences in regeneration rate between laboratory and field because of more nutrients being available in the field to fuel regenerative processes.

Oxygen availability also affects regeneration rate. In the brittlestar species *Amphiura filiformis*, regeneration rates were decreased in hypoxic conditions compared to normoxia (Nilsson & Sköld, 1996). In the laboratory, there were one or two days where there were

issues with sustained seawater flow, which may have influenced the regeneration time through decreasing the oxygen available to the animals. In the field, the animals were subject to tidal movements and thus, there was always an influx of fresh seawater twice a day with the incoming tide. It is therefore possible that differences in oxygen availability between the laboratory and the field existed and may have impacted regeneration rates.

2. 4. 2. Respiratory tree regeneration

Across all three experimental groups (6dl, 10dl, and 10df), the respiratory tree was consistently one of the first organs to begin regenerating. Sixty-six percent, 75% and 100% of whole excised animals in the 6dl, 10dl and 10df groups had regenerating respiratory trees, respectively. This regeneration rate was similar to that described for *H. scabra* and *A. japonicus*, where *H. scabra* begins respiratory tree regeneration one-day post-evisceration and *A. japonicus* displays 15-20 mm of respiratory tree growth 15 days post-evisceration (Bai, 1994; Dolmatov & Ginanova, 2009). Respiratory trees are the primary breathing organ of sea cucumbers, where oxygen is extracted from the water that flows across them. The mechanism by which respiratory trees regenerate following evisceration has been described for *A. japonicus*, where wound healing, followed by the formation of respiratory tree rudiment and cell differentiation occurs as a result of cell dedifferentiation, migration and proliferation (Dolmatov & Ginanova, 2009). The speed at which an organ is regenerated is a reflection of its functional importance (Needham, 1952). Therefore, relatively fast regeneration of the respiratory tree is likely reflecting its functional importance in maintaining efficient oxygen uptake, because without this organ, oxygen uptake is less efficient (Zang et al., 2012).

The function of the respiratory tree is not only limited to oxygen uptake. A study on the sea cucumber *Parastichopus californicus* showed that respiratory trees are responsible for a higher amount of free amino acid uptake compared to other tissues (Brothers et al., 2015). It has been suggested that these organs have a role in nutrient uptake (Brothers et al., 2015), and this could be particularly important in eviscerated or excised animals when nutrient uptake from digestion is impaired. It is likely that the speed with which the respiratory tree regenerated is a reflection of its role in both oxygen and nutrient uptake, and that it may be the organ which facilitates free amino acid uptake during periods of organ regeneration. A pattern by which respiratory tree and intestinal regeneration occur relatively simultaneously

has been demonstrated in *A. japonicus* (Dolmatov & Ginanova, 2009) and it is likely that a similar pattern is present in *S. horrens* (**Figure 2.4, Figure 2.5 & Figure 2.6**).

2. 4. 3. Gonad regeneration and development

No excised animals showed any visible signs of gonad regeneration in any of the groups (6dl, 10dl and 10df) (**Figure 2.4, Figure 2.5 & Figure 2.6**). It is likely that the time-frames here are too short for gonads to be regenerated which is unsurprising given the wide acceptance that gonads regenerate after all other major internal organs (Drumm & Loneragan, 2005). This has been demonstrated in *Holothuria leucospilota* (Drumm & Loneragan, 2005), *Thyone briareus* (Kille, 1935), and *Holothuria parvula* (Kille, 1942), with *H. leucospilota* taking longer than 40 days to regenerate gonads (Drumm & Loneragan, 2005). Gonads (when present) were very small and found only in sham and control treatments indicating that they were present before the experiment began. The sexually reproductive season of *Stichopus horrens* is between January and March each year (Muthiga et al., 2010). The low frequency and small size of gonads described here is likely because the current experiment was conducted in winter (May – June). The lack of gonads is likely due to gonad resorption - a phenomenon known to occur in sea cucumbers following the sexual reproductive season (Kille, 1942). Given that all controls had both full intestines and a respiratory tree, it is unlikely that they had recently been fished and had their gonads removed. Therefore, gonad resorption is the most likely explanation for the lack of gonads seen in these animals.

It has been demonstrated that the loss and patterns of gonad regeneration following evisceration is partially a consequence of the season (García-Arrarás & Greenberg, 2001). Given that *S. horrens* is believed to be sexually reproductive in the summer, I would suggest conducting a similar experiment to this over a variety of seasons and over a longer time period, to determine if the lack of gonad regeneration seen here is a consequence of season and/or insufficient time for gonad regeneration.

2. 4. 4. Asexual reproduction

S. horrens can reproduce both sexually and asexually (Dolmatov, 2014). The capacity for *S. horrens* to asexually reproduce via fission (dividing in half) was demonstrated in the current study. The highest rate of budding was shown in the excised treatment in the 10df group (**Figure 2.7**). It has been demonstrated in *Holothuria atra* that a relatively low rate of asexual reproduction is maintained throughout the year (Thorne et al., 2013) and it is likely that the controls in the field group are an indication of a similar reproductive pattern in *S. horrens*.

The frequency of budding demonstrated here is much higher than that described for *H. leucospilota* during its sexually reproductive season (Drumm & Loneragan, 2005). Therefore, it is possible that the relatively high proportion of budding that was shown in this study is a consequence of the season (this study was conducted in the Tongan winter, the non-reproductive season). In studies of *H. atra* the highest proportion of asexual reproduction was demonstrated in winter and spring (Lee et al., 2008b). Another example of a correlation between winter and increased fission rate has been seen in the fissiparous sea star *Nepanthia belcheri* (Ottesen & Lucas, 1982). It should be noted, however, that traditional fishing does still occur during the Tongan winter, and therefore the rate of asexual reproduction demonstrated in the excised group is likely to be seen over this season in areas under high fishing pressure.

The present experiment suggests that both excision and sham treatments may potentially act as a stimulus for budding (**Figure 2.7**). To the best of my knowledge, no studies investigating the effects of excision following traditional fishing techniques on the fission rate in sea cucumbers have been conducted. However, it does appear that abiotic factors such as a harsh or resource-depleted environment, high densities of individuals, and season, can stress the animals and influence the proportion of asexual reproduction in echinoderms (Mladenov, 1996). Given that excision and sham treatments are likely to represent a stressful event, it is possible that this stress has been sufficient enough to cause elevated fission rates in these treatments. Fission may also act to reduce the surface area to volume ratio as a mechanism to facilitate more efficient oxygen diffusion across the body surface (Gillooly et al., 2001). This is not supported by the MO_2 results (**Figure 2.8**) or the positive significant relationship between animal size and MO_2 (**Figure 2.12**) which suggests that MO_2 increases with body size.

In the current study, the highest proportion of budding occurred in the laboratory. This may be due to differing experimental conditions or random effects, but there is also the possibility that the laboratory animals were experiencing environmental stress due to their ‘unnatural’ holding conditions. Furthermore, an absence of sediment in the intestinal tract in any of the laboratory animals at either 6 or 10 days indicates that the animals may not have been feeding during this study. The relatively low body wall glycogen levels also suggest that the animals utilised glycogen stores as an alternative energy source and were not feeding during the experiment (**Figure 2.17**). It has been suggested that food supply is related to fission, with lower rates of fission being a reflection of high food abundance (Sköld et al., 2002). Given

that it appears that laboratory animals were not feeding; it is possible that the increased rate of fission in the laboratory compared to the field may have been due to starvation. However, as discussed above, it is possible that *S. horrens* was feeding but that the time when sediment is in the intestinal tract did not coincide with the time that dissections occurred in the present study (Eriksson et al., 2007).

The increased frequency of budding due to traditional fishing techniques (excision) may have implications for sexual reproduction. Asexual reproduction can delay gonad development (Kille, 1942). Therefore, the length of time it takes for *S. horrens* to regenerate fully functional gonads following excision should be investigated. This timeframe should then be included in the development of a traditional fishing ban to allow sufficient gonad development before the reproductive season to ensure the maintenance of natural sexual reproduction levels. However, a ban on traditional fishing (i.e. collecting raw internal organs from the sea cucumbers) is unlikely to be feasible because many local communities rely on this as a food and income source. Therefore, it should be investigated whether *S. horrens* can still sexually reproduce following the removal of all organs except gonads. This would indicate more definitively whether sexual reproduction is negatively affected by internal organ removal.

2. 4. 5. Physiology: Oxygen consumption rate and ammonia excretion rate

Whole animals had a higher MO_2 than both anterior and posterior buds (% of overall treatment mean) (**Figure 2.8**). It is widely accepted that mass-specific MO_2 increases with animal size (Reichle, 1968; Sebens, 1981; Heusner, 1982; Sebens 1982; Speakman, 2005). In the class holothuroidea, a significant positive correlation between mass and MO_2 has been demonstrated (Hughes et al., 2011). **Figure 2.12** also demonstrate this, with larger animals having a greater mass-specific- MO_2 than smaller sea cucumbers. Given that the buds were much smaller than the whole animals (mean \pm standard errors; buds: 15.89 ± 1.8 g, whole animals: 28.1 ± 2.24 g), the difference observed in MO_2 between buds and whole animals is likely to be due to differences in body weight and relative organ size.

A reduced MO_2 in field excised sea cucumbers compared with the control animals (**Figure 2.11**) is aligned with other findings in eviscerated sea cucumbers such as *A. japonicus* and *Stichopus parvimensis* which also demonstrated a drop in MO_2 following evisceration (Dimock Jr, 1977; Zang et al., 2012). Sea cucumbers are able to obtain oxygen via both the respiratory tree and by diffusion across the body wall, but respiration via the respiratory tree

is more efficient than oxygen consumption by body wall diffusion (Choe 1963 in Zang et al., 2012; Zang et al., 2012). Therefore, the lower MO_2 in excised animals compared to control sea cucumbers is quite possibly due to the lack of (or the reduced size of) the primary breathing organ (the respiratory tree) in the excised animals.

The 10dl group had a higher oxygen consumption rate than the 6dl group (**Figure 2.10**). The increase in MO_2 over time following evisceration has been demonstrated in the species *A. japonicus* (Zang et al., 2012). This persists until the animal is believed to have recovered from evisceration and regains a 'normal' MO_2 . It is likely that the increase in oxygen consumption rate demonstrated from 6 to 10 days would continue until the animal had fully regenerated its breathing apparatus and other internal organs and 'recovered' from excision.

The whole excised 10df animals had a lower MO_2 than the whole excised 10dl animals (**Figure 2.11**). Given that 100% of field animals and 75% of laboratory animals had a respiratory tree, it seems unlikely that there should be such a marked difference between laboratory and field excised animals. It is likely that this difference represents the difference in holding conditions between these two experiments, as field collected animals are known to change their physiological parameters in response to laboratory conditions (Ikeda, 1977). Furthermore, laboratory conditions have been known to affect physiological parameters such as excretion and respiration rate in marine invertebrates (zooplankton) (Ikeda, 1977) as well as regeneration time in sea cucumbers (García-Arrarás & Greenberg, 2001).

In this study, the rate of nitrogen excreted as ammonia was measured and it was shown that there was no significant difference in AER between budded and whole animals (**Figure 2.13**). A study by Uthicke (2001a) also demonstrated this result in *Holothuria atra*, and this author suggested that this was likely due to the capacity for these animals to excrete ammonia across the body wall via diffusion. There was a significant difference in AER between the 10dl and 10df groups; however, because this data was non-parametric, a two-way ANOVA to test the interaction between location and treatment could not be performed. It appears that the significant difference between the groups lay in the excised treatments, with the field excised group having a higher AER than the laboratory excised group (**Figure 2.15**). This may have been due to the higher availability of dissolved amino acids in the field than in the laboratory (See section 2.4.1.).

2. 4. 6. **Biochemistry: Glycogen, O:N, Glucose, and Lactate**

The O:N can be used to explain why there were differences in body wall glycogen content between groups and treatments. The average O:N for whole animals in the control, excised and sham treatments of the 10dl group fell between 15 and 32 (**Figure 2.18**). O:N values greater than 8 indicate that a combination of proteins and lipids are being used as the primary source of energy (Ikeda, 1977; Chandurvelan et al. 2012). Higher values (such as those for the control treatments in the 10dl group; **Figure 2.18**) are indicative of carbohydrate and lipid use (Ikeda, 1977; Chandurvelan et al. 2012). Thus, in the 10dl groups, the average O:N suggests that these sea cucumbers were relying on a combination of proteins, lipids and carbohydrates for the energy required to fuel life processes and tissue regeneration. Given that they are unlikely to be actively feeding (due to the lack of an intestine) the carbohydrates for regeneration are likely being supplied by the body wall. This theory is supported by evidence in the literature which indicates that holothurians are able to obtain nutrients for organ regeneration from morula cells and amoebocytes which carry nutrients from the body wall to the intestines via the mesentery (connective tissue attaching the body wall to digestive tract) (Dawbin, 1949; Bai, 1994). It is possible that the sea cucumbers could be obtaining nutrients via the respiratory tree (Jaekle & Strathmann, 2013), however to the best of my knowledge there is no research which indicates at which stage during regeneration the respiratory tree can function in nutrient uptake.

By comparison, on average, the lowest O:N was demonstrated in the field group. For the control and excised treatments, the average O:N was 14 and 4 respectively. Compared to the higher values per treatment displayed in the laboratory group, this indicates that the field sea cucumbers were less reliant on carbohydrate/lipid metabolism than the laboratory group (**Figure 2.18**). The excised sea cucumbers, with an average O:N of 4, are relying on protein as a primary energy source; with O:N values less than 7 indicating that only protein is being used (Zhao et al., 2014). As discussed above in Section 2. 4. 1., this is likely a reflection of likely greater free amino acid availability in the field compared to the lab group. The relatively higher glycogen body wall concentrations in the field group compared to the laboratory group (**Figure 2.17**) is likely a reflection of the sea cucumbers using different primary energy sources.

In the 10df group, excised animals had higher glycogen stores in the body wall than controls (**Figure 2.17**). The O:N indicates that controls are utilising both carbohydrate/lipid and

proteins, while the excised animals are using proteins only. Therefore, the controls are depleting the carbohydrate stores in their body walls (glycogen) faster than the excised animals because they are using different energy sources. The controls may not have been feeding in this experiment as shown by both the glycogen body wall concentrations and the lack of sediment in all but one animal's intestines, and it is possible that starvation may have resulted in the depletion of the body wall glycogen reserves for energy. This has been demonstrated in an Antarctic krill species, *Euphausia superba*, in which starvation resulted in a 30% decrease in glycogen body stores over a starvation period of 18 days (Auerswald et al., 2009). The energetic cost of maintaining functioning organs in the controls may have been higher than that in the excised animals which had very few internal organs to maintain. Although they were regenerating tissues, which is a process likely to have its own energetic costs, perhaps the process of regeneration requires more protein than carbohydrates. Protein synthesis is responsible for the repair stage of regeneration (Needham, 1952), therefore the relatively higher use of proteins in excised animals compared to controls is likely reflecting an elevated requirement of protein to fuel regenerative processes. Additionally, it is likely indicating that the excised animals at 10 days are largely in the repair phase of regeneration.

In the present study, both the tissue and coelomic fluid lactate levels were very low, and below the sensitivity of the assay procedure used (See Section 2.3.4.1.). The low lactate levels reported in this study fall within the scope of those measured in other marine invertebrates such as the crab *Chionoecetes tanneri* (with hypoxia having no effect on lactate levels) and *Cancer magister* (for which baseline lactate production was reported as being just above 0 mM; Pane & Barry, 2007). Under normoxic conditions, the toad *Bufo marinus* demonstrates plasma lactate levels close to 0 mmol L⁻¹ (Pörtner et al., 1991; Rose et al., 1998).

Lactate is the end-product of anaerobic metabolism. The function of the lactate pathway is to elevate the production of energy to fuel muscular work under anaerobic conditions (Livingstone, 1983). Anaerobiosis is a less efficient energetic pathway than aerobic respiration (Hardy & Briffa, 2013). As a result, an oxygen debt is incurred via anaerobic pathways, and this debt refers to the quantity of oxygen required to convert lactic acid to CO₂ and water (Hardy & Briffa, 2013). In the velvet swimming crab, *Necora puber*, the avoidance of anaerobic metabolism is thought to be due to the high energetic costs associated with anaerobiosis (Thorpe et al., 1995). Additionally, a reduced arm regeneration rate in the brittlestar *Amphiura filiformis* under hypoxic conditions compared with normoxic conditions

has been demonstrated (Nilsson & Sköld, 1996). Thus, anaerobic metabolism is energetically costly, and given that regeneration represents an energetic cost (Nilsson & Sköld, 1996), anaerobiosis is likely to have an effect on regeneration rate. Taken with the results from this study which suggest that regeneration falls within the aerobic scope of the sea cucumbers, it is likely that they avoid anaerobiosis because it is energetically costly, and it is possible that this is a method by which to conserve fuel for tissue regeneration.

There was no significant effect of treatment or location on the body wall glucose concentration (**Figure 2.16**). In the 10dl group there appeared to be higher glucose than glycogen in the body wall. Given that glycogen can be readily broken down into glucose for fuel, it is not unlikely that the lower body wall glycogen relative to glucose is an indication of glycogen metabolism (Berg et al., 2002). In the 10df group, the body wall glucose was lower than the glycogen indicating that glycogen reserves were not being depleted through metabolism. It is possible that starvation in the 10dl group may be responsible for the decrease in glycogen stores and the increase in glucose to provide fuel (Auerswald et al., 2009). This is supported by the O:N which suggested more carbohydrate based metabolism in the laboratory groups than in the field on average (**Figure 2.18**) (Ikeda, 1977; Chandurvelan et al., 2012).

2. 4. 7. Summary

This study revealed that the internal organs that regenerate first following excision are the respiratory tree and the intestine, which is likely due to their functional importance in feeding and oxygen uptake. Given that gonads did not regenerate, and that the literature suggests that gonad regeneration is a relatively slow process, this study may have implications for managing traditional fisheries. Dissections also showed that very few individuals possessed gonads during the time period of May to June, which indicates that *S. horrens* does not sexually reproduce during this time. It also presents a possibility that this species may resorb gonads when it is not their sexually reproductive period.

This study also suggests that holding conditions may have an effect on the AER, MO_2 , and the primary energy source utilised by the animals. It is possible that this may have implications for future aquaculture practices concerning *S. horrens*. This study also highlights the potential importance of dissolved nutrients for sea cucumbers during periods of starvation (internal organ regeneration), and indicates that sea cucumbers are able to utilise the carbohydrate stores in their body wall when necessary. This may also have important

implications for aquaculture practices, whereby it may be important to take into account dissolved nutrient inputs as well as sediment nutrient inputs when considering optimal sea cucumber diets.

This study also showed that *S. horrens* is capable of asexual reproduction, and that it is possible that excision (under field conditions) may act as a trigger for asexual reproduction. Sexual reproduction is important in that it promotes genetic diversity, thus a finding of enhanced asexual reproduction, which will slow sexual development, may have implications for the management of this particular traditional sea cucumber fishery. It appears that excision results in a decreased metabolic rate compared to control animals (in the field group). This is likely a reflection of reduced oxygen extraction efficiency following respiratory tree removal, but could also be indicative of a decrease in metabolic rate following excision to conserve energy for regeneration. I would suggest that in future studies a third treatment whereby the intestines alone are taken out be administered which would perhaps clarify this relationship. Interestingly, regeneration falls within the aerobic scope of *S. horrens* which indicates that the sea cucumbers were able to avoid the costs of anaerobic respiration, which may have been an energy conservation mechanism.

Taken together, the results of this study indicate that internal organ removal and subsequent regeneration present an energetic cost for these animals, particularly in systems which may be low in dissolved nutrients. It highlights that delayed gonadal development is likely to be the biggest cost of regeneration given that gonads were not one of the first organs to regenerate.

3. The Ecological Distribution and Diversity of holothuroids of the Tongatapu Island group

3.1 Introduction

3.1.2. Sea cucumber ecology

Sea cucumbers are remarkably diverse marine macroinvertebrates - with 187 species recorded in the Antarctic region alone, and with higher species diversity recorded in tropical systems compared with those of cooler regions (Conand, 1993b; O’Loughlin et al., 2011). They occupy a wide array of marine habitats, from seagrass beds (Taquet et al., 2011) to deep-sea (Barnes et al., 1976). Their life cycle includes an egg, larval and juvenile stage, and from the juvenile stage it takes 2 – 3 years of growth before they reach sexual maturity (Friedman et al., 2008c).

Seagrass beds and coral reef systems are very important components of the marine environment. For example, reefs provide habitat for juvenile fish species such as *Lutjanus mahogany* and *Acunthurus bahianus* (Nagelkerken et al., 2000) and these environments harbour a large amount of the ocean’s biodiversity (Pandolfi et al., 2011). Seagrass beds are a source of organic carbon, and they act to stabilise sediments and to provide shelter and habitat for a diverse suite of marine organisms (Orth et al., 2006). Seagrasses can also represent important nursery areas for juvenile sea cucumbers (Taquet et al., 2011) and relatively high settlement rates on seagrass compared to other substrates have been demonstrated in *Holothuria scabra* (Mercier et al., 2000b). Research has shown that sea cucumbers may play an important role in seagrass productivity (Wolkenhauer et al., 2010). Additionally, they are also vital components of reef systems where they help to maintain calcium carbonate balance (Eriksson et al., 2012).

The feeding behaviour of sea cucumbers is an essential component to the functioning of the reef and seagrass environments. Their feeding behaviour can range from suspension feeding

whereby they capture food particles from the water column, to deposit feeding whereby they ingest sediment (Graham & Thompson, 2009). A study by Schneider et al. (2011) demonstrated that the break-down of sediment through the digestive tract of sea cucumbers causes calcium carbonate to dissolve, which can in turn increase the alkalinity of seawater. Thus, the dissolution of calcium carbonate via the feeding activity of sea cucumbers may assist in buffering marine systems against ocean acidification (Schneider et al., 2011). Additionally, it has been suggested that sea cucumbers are involved in benthic recycling; the ammonia waste excreted by these animals can act as a nutrient which promotes macroalgal growth and offset the impacts of sea cucumber grazing on microalgal populations (Uthicke, 2001a, b). Thus sea cucumbers play a key role in nutrient cycling in the marine system.

Not only do sea cucumbers cycle nutrients, but they can also act as a buffer against excessive nutrient loading. In co-culture with the sablefish *Anoplopoma fimbria*, the Californian sea cucumber *Parastichopus californicus* was able to significantly reduce the organic load of the fish's faeces and thus, reducing the organic nutrient input into the marine system through their feeding behaviour (Hannah et al., 2013). Sea cucumbers can also act to reduce the effects of pollution from mussel farms by assimilating organic nutrients, highlighting how effective their feeding behaviour is at buffering against nutrient pollution (Slater & Carton, 2009). It is also possible that they can mitigate human waste pollution. A stable isotope analysis showed that benthic deposit-feeding sea cucumbers *Benthodytes sanguinolenta*, had assimilated sewage derived organic matter at a deep-water sewage dump-site off the coast of New Jersey (Vandover et al., 1992). Thus, sea cucumbers are very important in the marine nutrient cycle, potentially acting as a buffer against nutrient loading and ocean acidification.

Sea cucumbers are also an important part of the food web, not just as nutrient cyclers and sediment cleaners, but also as prey items for other marine species. Aside from humans, sea cucumber predators include; the sea star *Solaster endeca* (So et al., 2010), nearshore crab species (personal observation, Tongatapu, November, 2014), the crab *Thalamita crenata* (Lavitra et al., 2015) and fish which are known to prey on the eggs and juveniles of sea cucumbers (Iyengar & Harvell, 2001). It has been shown that injury may affect predation rate, with injured specimens of the sea cucumber *Cucumaria frondosa* being preyed upon more frequently by *S. endeca* than those which were not injured (So et al., 2010). This suggests that injuries sustained through the use of traditional fishing techniques and other human activities such as trawling, may influence predator-prey relationships in marine food webs.

3. 1. 3. Ecological surveys/stock assessments

Biodiversity assessments are an important data source for informing management decisions (Heywood, 1995). They can provide information about the abundance and diversity of species, and if these ecological surveys are carried out over time, can indicate how these might change long-term. Friedman et al. (2008a), suggested that the use of manta-tow boards is the best technique for conducting sea cucumber surveys. The manta-tow technique has been widely used in many sea cucumber surveys in the Pacific over the last 10 years, and consists of a manta-board being towed behind a boat with a snorkeler or diver attached who records the data. This method has been employed extensively by Awira and colleagues (2008) to assess sea cucumber populations in Kiribati, for example. These authors conducted broad-scale assessments using 300 m × 2 m wide transects in inshore, midshore and oceanic habitats, and also conducted fine-scale assessments using 40 m × 1 m wide transects in selected areas of reef and soft-benthos (Awira et al., 2008). In addition, SCUBA equipment was also used to conduct deep water dives in the surf zone (25 – 35 m deep) to assess deeper sea cucumber stocks. Night surveys of inshore reefs were also carried out to investigate species displaying nocturnal activity (Awira et al., 2008). In addition to this Kiribati study, these exact techniques were used in 17 other Secretariat of the Pacific Community (SPC) reports for individual countries, including Tonga, between 2007 and 2016 (literature search of the SPC Digital Library filtered by sea cucumber in ‘field’ and survey in the ‘title’ across information bulletins, fisheries newsletter, Beche-de-mer information bulletin, traditional management, and papers and reports) (Awira et al., 2008, Friedman et al., 2009a,b, Friedman et al., 2008a,b, Friedman et al., 2010, Kronen et al., 2009a,b, Kronen et al., 2008a,b,c, Pinca et al., 2009a,b,c, Sauni et al., 2008, Sauni et al., 2007, Vunisea et al., 2008).

Different sampling regimes i.e. broad vs. fine-scale can be better suited to informing different aspects of community composition and cover (Fonseca et al., 2002). In addition to manta-tow surveys, the use of transects and quadrats to conduct ‘fine-scale’ abundance assessments can be employed (Eriksson, 2006). These techniques have been used in a number of studies on holothuroid abundance (Kerr et al., 1993; Woodby et al., 2000; Shepherd et al., 2003; Lawrence & Sonnenholzner, 2004; Pouget, 2005; Hasan, 2009). Fine-scale assessments are able to provide more accurate information about cryptic species, and can sometimes be a more reliable species identification and count method compared to timed swims and, by proxy, manta-tow surveys (Obermeyer, 1998).

In Tonga, sea cucumber stocks have been reasonably well documented since 1984 (Pakoa et al., 2013). A sea cucumber survey was conducted in 1996, in which divers counted sea cucumber abundance in deeper areas, and walked transects on reef flats (Lokani et al., 1996). The survey showed that the sea cucumber stocks of the white teatfish (*Holothuria fuscogilva*), the black teatfish (*Holothuria nobilis*), the prickly redfish (*Thelenota ananas*), and the amberfish (*Thelenota anax*), had been depleted from pre-fishing to post-fishing times (Lokani et al., 1996). The conclusion was made that the stocks were depleted and as a direct consequence of these surveys, a 10-year moratorium on commercial export fishing was put in place. In 2009, the Tonga Country Report, having conducted sea cucumber surveys (as outlined above for the reports of individual countries), showed that the majority of sea cucumber stocks in the Ha'atafu, and Manuka regions had still not recovered following the moratorium, however, in the Lofanga and Koulu regions some stocks had recovered to the point where conservative commercial fishing could have been viable (Friedman et al., 2009c). In 2010 another stock assessment was conducted, and it was suggested that a three to five-year ban be put on commercial fishing (Pakoa et al., 2013). This ban was protested by exporters and as a result, the sea cucumber fishery remained open until 2015 (Pacific Islands Development Project, 2014). In 2015, a five-year ban on commercial fishing was proposed, and again met with opposition from fishers. However, the Tongan government approved the ban, which is currently in place (pers. comm. T. Halafihi, 11.02.2016).

Results of an underwater visual census stock assessment in Vanuatu, presented by the Vanuatu Fisheries Division (2011) suggested that even 5 years following a moratorium (imposed in 2007) on sea cucumber harvesting, the size and number of these animals was still small (Nimoho et al., 2013). They suggest that this is likely due to a combination of the slow growth rate of sea cucumbers and the continuation of fishing (illegally) during the moratorium (Nimoho et al., 2013). Therefore, to ensure that the 5 year fisheries ban in Tonga is successful, and to prevent the legalisation of commercial fishing before sea cucumber stocks have recovered, it is important to monitor sea cucumber abundance and diversity.

To provide ecological information for the Tongan Fisheries Division to assist in informing nearshore sea cucumber management decisions, a series of ecological surveys were carried out in the nearshore environment around Tongatapu in the present study. The goal of this research was to provide baseline ecological data to the Tongan Fisheries Division, and to better understand the ecological factors which may encourage sea cucumber abundance and diversity at various sites around Tongatapu.

3. 2. Methods

3. 2. 1. Site identification

Four sites were identified around the island of Tongatapu (**Figure 3.1**), based on their differing levels of human activity and perceived fishing pressure. Three of these sites were on the main island of Tongatapu and are hereon referred to as onshore sites. Two of these sites (Tukutonga and Sopus) were situated close to the port and very close to the Tongan capital city, Nuku'alofa, and were areas with intense local fishing pressure and relatively high boating traffic. The third onshore site of Kolonga was at the edge of the lagoon. This site was chosen because it was of special interest to the Tonga Fisheries Division, who wanted to identify the levels of sea cucumber resource depletion to potentially review the management of the area. The fourth site, the offshore island of Atata was chosen to represent a location of low human activity with reduced fishing pressure.



Figure 3.1. Image from Google Earth displaying the four sampling sites (in orange font) around the island of Tongatapu in the Kingdom of Tonga, including the offshore island of Atata, the capital of Tonga; Nuku'alofa, and the village of Manuka which bordered the site of Kolonga.

At each of the four sites, two stations were chosen based on accessibility and distance from each other to get as much coverage of the site as possible. Two stations were chosen per site

because of the trade-off between time and logistical constraints. Furthermore, it was necessary to sample a large enough area that any sea cucumber abundances sampled were representative of the wider area. The distance between stations within each site was approximately 500 m at Kolonga and Sopu, and approximately 100 m at Atata and Tukutonga. The differences in distance between each station within sites was to account for differing site characteristics.

Atata was the only offshore island sampled, and the two stations which were chosen to be sampled at Atata were selected based on their ease of access and their management regime. It was also the only site for which each side of the island was sampled. Atata Station-1 (AT1) was situated on the side of the island (east) that faced the town of Nuku'alofa, whereas Station-2 (AT2) was west of the island and was within a Special Management Area (SMA) meaning that only people from the village of Atata were allowed to fish there.

3. 2. 2. Ecological sampling

At each station, habitat was characterised into either 'Sargassum/rubble dominant' or 'Seagrass dominant' habitats. To classify as 'dominant' the dominant cover needed to be the feature characteristic (i.e. > 50 %) of the habitat. Sargassum is a brown macroalgae (seaweed) in the order Fucales and was often found in zones of 'rubble' (loose rocks and large pieces of gravel (> 5 cm in diameter) found in the back-reef area), with these two features combining to form the dominant cover. Seagrass is a marine flowering plant often found growing in large meadows or beds, and was found in the nearshore environment around Tonga. Sampling events occurred over two seasons, November – December 2014 and May – July 2015. The two seasons were sampled because they coincided with the timeframe preceding the implementation of the commercial fishing ban, and the timeframe following its recent commencement. Within each habitat, two sampling events per season were carried out, one at night and one during the day to determine if sea cucumber abundance or species present changed diurnally.

A preliminary sample was done at Sopu reef to determine the optimal number of quadrats required for accurate estimation of sea cucumber abundance. At this site, four 10 m transects were sampled in the sargassum/rubble habitat and three 10 m transects were sampled in the seagrass habitat. Only three transects were sampled in the seagrass habitat at this sampling event because of tidal time constraints. The transects were laid 15 steps apart, and at each 2 m mark along each 10 m transect, a 1 m² quadrat was placed on each side to get a total of 10

quadrats per 10 m. A cumulative distribution plot (**Figure 3.2**) of these data showed that 40 was a robust number of quadrats to sample and get a true representation of the sea cucumber population numbers. Based on the cumulative distribution plot, the observation that sea cucumber distribution was patchy, and combined with logistics and time constraints of low-tide sampling, 40 quadrats were decided as the sample unit. At each sampling event following the preliminary survey (with a sampling event being defined as one habitat within one station within one site within one sampling season – 2014 or 2015), eight 10 m transects were laid out at regular intervals throughout the habitat and 1 m² quadrats were placed on alternating sides every 2 m along the transect. The number of steps between each transect was adjusted to be proportional to the width of the band of seagrass at each site. For example, Sopu reef had a very wide seagrass band stretching approximately 200 steps from the shore while Tukutonga had a relatively narrow seagrass band and so the number of steps between each transect was different between the sites, but was kept consistent within the sites. In total, eight transects were laid per habitat, all perpendicular to the shore to reduce the potential for confounding variables and covariates.

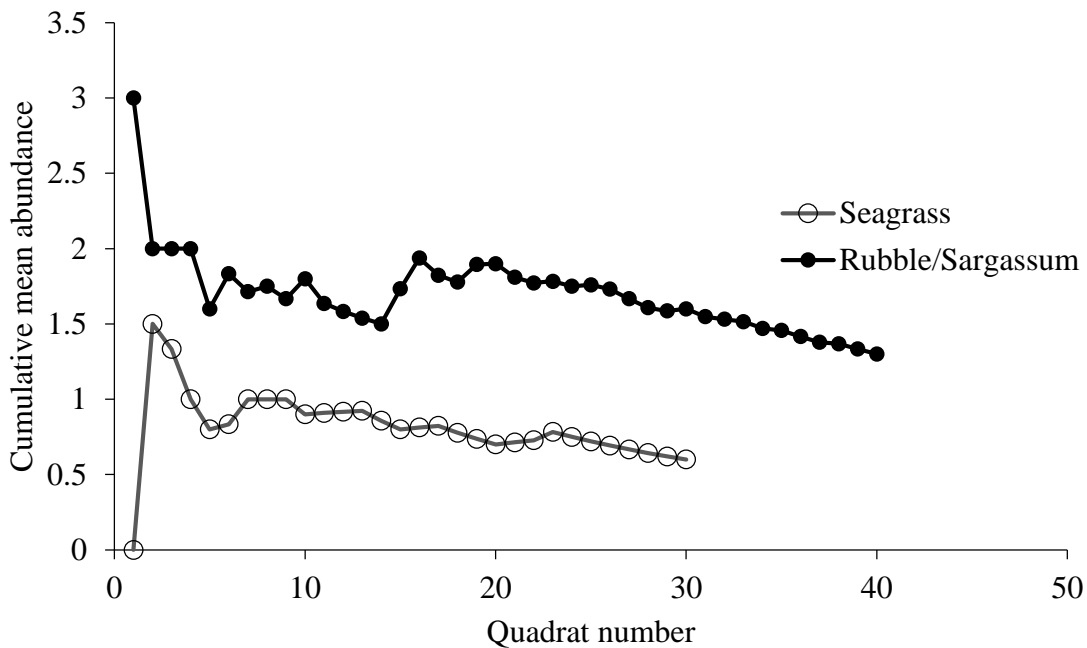


Figure 3.2. Distribution plot displaying the cumulative mean sea cucumber abundance as a function of the number of quadrats at Sopu reef in the sample season 2014 at night for both the rubble/sargassum and seagrass habitats.

Within each quadrat, the habitat cover (%) was recorded, along with the number of sea cucumbers, the species and each individual sea cucumber’s length (cm). For each quadrat, I

ran my hand systematically along the base of the seagrass because often visually identifying sea cucumbers was not reliable in low visibility water, and foraging for them in this manner returned a significant number of sea cucumbers as opposed to relying on visual counting alone. After each sea cucumber had been counted and measured in each quadrat, it was set down outside the quadrat to prevent it being re-sampled. Additionally, the water depth (cm), light intensity (Lux) and temperature (°C) for each quadrat was measured. This was done by attaching a HOBO data logger to the base of the depth stick and placing the stick in the centre of the side of the quadrat closest to the transect and leaving it there for 30 seconds. The GPS coordinates for each transect in the 2014 sampling season were also noted using the Navionics Pacific and Asia boating iPhone application. The habitat variables measured in each quadrat were: macroalgae, sand, rubble, pavement, live coral, dead coral, seagrass, soft coral, sponge, unknown sediment, calcareous algae, sargassum, padina, and blue-green algae (**Table 3.1**).

Table 3.1. Habitat features measured in this study and their descriptions.

Habitat feature	Description
Macroalgae	Fleshy macroalgae with a canopy height > 1 cm, defined separately to <i>Sargassum</i> and <i>Padina</i>
Rubble	Loose rocks and large pieces of gravel (> 5 cm in diameter) found predominantly in the back-reef area and on occasion in the seagrass
Pavement	Smooth rock substratum which was not loose like the rubble
Seagrass	Marine flowering plants often growing in large meadows or beds
Sponge	An unidentified species of marine sponge with an orangey cylindrical appearance which was often found in small aggregations on the seagrass bed
Unknown sediment	This was a component of the sand/sediment, with an oval and flat appearance. It is possible that this was a coralline alga but was unidentifiable.
Calcareous algae	Green macroalgae with a rigid structure, most likely of the genus <i>Halimeda</i>
Sargassum	A brown macroalgae (seaweed) in the order Fucales
Padina	A brown alga which has curled branches attached to a single stalk
Blue-green algae	This was the loose term applied to the brown film covering the marine sediment and many plants at the sample sites, and was most likely an algae resulting from nutrient loading

The goal of the present study was to gain an understanding of the species present, their densities, sizes and the factors which may be affecting their distribution. The combination of transects and quadrats were used to facilitate this, and thus the use of Manta tows and belt transect methods, which are considered useful for larger-scale stock assessment approaches, was not considered appropriate for the current study. This fine-scale approach was designed to ensure findings of this study were appropriate from an ecological perspective.

3. 2. 3. Data analysis

Temperature and light intensity was averaged for the first 20 seconds of data collection within each quadrat. This was done to reduce post-sampling processing and to reduce the influence of researcher interference within the quadrats. To determine if time of day, stations and sampling years were significantly different from one another, Kruskal-Wallis tests, following the use of the Shapiro-Wilk and Bartlett tests to determine normality and homogeneity of variances, were carried out on *Stichopus horrens* abundance data for each site. If there was no significant difference between time-of- day, then the time of day data within a station within a site was combined under the general heading 'Station'. If there was no significant difference between stations within a year, then the two stations were combined and treated as a 'year' sampling event. The effect of year on *S. horrens* abundance was then tested.

No outliers were removed from the dataset in the current study. This was because the data was zero-inflated (many quadrats were empty and thus sea cucumber abundance was zero) and outlier removal in such datasets resulted in a mean of zero, which would have incorrectly suggested that some sites had no sea cucumbers. Therefore, it was decided that outliers would not be rejected from any of the data sets because it could cause false inferences to be made from the data. When raw data did not pass the normality test the following transformations were trialled and each was tested for normality and homogeneity of variances: square root, $\log_{1p}(\log x + 1)$, \log , $\sqrt{x} + 0.5$, and arcsine. If the transformations did not normalise the data, then whichever dataset (transformed or untransformed) passed the Bartlett's test of homogeneity of variances was analysed using a non-parametric test (Kruskal-Wallis).

The statistical package RStudio (v0.99.892) was used to construct non-metric multidimensional scaling (NMDS) plots that were used to visualise differences in sea cucumber species abundances and habitat composition (seagrass habitat only to keep the analysis balanced) between the four different sites. NMDS was used because it is robust to data that does not have an identifiable distribution or a non-linear distribution (Oksanen, 2015), and it is able to handle multiple different data types (categorical and continuous data) as in this study.

To construct an NMDS plot for sea cucumber diversity, the average sea cucumber abundance was calculated for each species at each sampling unit. A sampling unit is defined as a sampling effort. For example, A1D14 (Atata site one day-time sampling 2014) represents one

sampling unit. At each sampling unit, a total of 40 quadrats (1 m² each) were sampled. The number of each species encountered was summed to get a total number of each species. This total was then divided by 40 to get mean abundance of that sea cucumber species per m². For example, the total number of *Holothuria atra* counted at A1D14 day was divided by 40, and then the same was done for *Stichopus horrens* and so on. Due to the large amount of data, the mean abundance per m² for each species per sampling unit was used as the basic unit of analysis for the NMDS and Permutational Multivariate Analysis of Variance (or nonparametric multivariate analysis of variance) (PERMANOVA) analyses on sea cucumber abundance. For the habitat composition NMDS plot, the percent habitat per m² was calculated by dividing the total percent cover for a given component of the habitat (e.g., seagrass) and then dividing by the sampling unit (total number of quadrats).

Following the NMDS plot, a PERMANOVA using the Bray-Curtis dissimilarity matrix was conducted using the ‘adonis’ function in RStudio. Nine hundred and ninety-nine permutations were specified for each of these analyses. A PERMANOVA was chosen because this can be applied to non-normal and multivariate datasets (Oksanen et al., 2016). Therefore, because of the multivariate nature of the dataset and the ability of this technique to analyse any distance measure appropriate to the data set, it was chosen as the appropriate methodology. If the adonis test yielded a significant difference between sites, a similarity percentage (SIMPER) test was conducted to identify the species or habitat component that contributed the most to this significant difference.

Generalised linear models (glms) specifying a Poisson family error distribution for count data were used to determine which habitat and environmental variables had a significant effect on the abundance of *S. horrens* across all sites. *S. horrens* was chosen because this species was used in the regeneration experiments (Chapter II), and because it is fished for subsistence purposes in Tonga (Kinch et al., 2008). Generalised linear models were used because they allow non-normal response variables to be analysed, and are a flexible version of normal linear regression. In this dataset, the response variable, *S. horrens* abundance, is count data and the standardised residuals had a non-normal distribution as determined by a Shapiro-Wilk test ($W = 0.721, p < 2.2 \times 10^{-16}$). Given that the response variable was count data, a Poisson distribution was specified. Sites were included in the glm dataset regardless of whether or not any individuals of *S. horrens* were found there. This was because there may have been habitat or environmental variables influencing the lack of *S. horrens* at certain sites, which is equally as important to include in the model as the variables influencing high sea cucumber

abundance at other sites. Initially, all habitat and environmental variables were run in a glm with a Poisson distribution, and non-significant ($p > 0.05$) variables were removed sequentially. The interactions between the final significant variables ($p < 0.05$) were tested using a glm with a Poisson distribution.

3. 2. 4. Species richness

The species richness is the total number of species present at a sampling event. The average species richness for each site was calculated by averaging the total number of species present for each sampling event (year) at a particular site. This was to get a general indication of the differences in species richness between sites.

To determine if there was a significant difference in species richness between time-of-day within sites, a nested ANOVA was conducted. The results of a Shapiro-Wilk normality test on the standardised residuals of the untransformed species richness dataset was significant ($W = 0.926$, $p = 0.039$) when testing the distribution of the relationship between species richness and time of day (day vs. night). This indicates that the data may not strictly be normally distributed; however, no other transformations made this result any less significant. The results of a Bartlett test showed that the data was homogeneously distributed ($K = 0.847$, $df = 1$, $p = 0.358$). Given that ANOVAs are robust to slight deviations from normality as long as the data meets the assumption of homogeneity of variance (McDonald, 2014), it was decided that an ANOVA would be used here.

3. 2. 5. Shannon-Wiener diversity index

The Shannon-Wiener diversity index was used to measure the diversity of each community at each sampling event. This method was used because it allows for the exploration of both abundance (richness) and evenness (relative abundance). The Shannon-Wiener diversity index taken from Molles and Cahill (1999) was calculated using Equation 3.1:

Equation 3.1

$$H' = - \sum p_i (\ln p_i)$$

Where: H' is the Shannon-Wiener diversity index, and p_i is the proportion of individuals belonging to the i th species.

3. 2. 6. Evenness

The evenness index, E_{var} , was chosen based on the findings by Smith and Wilson (1996) in which E_{var} is the recommended general measure of evenness. Equation 3.2 was used to calculate E_{var} .

Equation 3.2

$$E_{var} = 1 - 2 \div \pi \arctan \{ \sum (\ln(x_s) - \sum \ln S)^2 / S \}$$

Where x_s is the abundance of a given species per m^2 per sampling event, x_t is the total abundance of all species per m^2 per sampling event, and S is the number of species present in that sampling event (i.e. species richness).

3. 3. Results

Four sites were successfully sampled in both 2014 and 2015. Overall, a total of nine identifiable and two unidentifiable species were found. In the seagrass habitat at each site, the number of species ranged from 1 to 6.

3. 3. 1. Differences in sea cucumber composition between sites – ‘Seagrass’ habitat

According to the Bray-Curtis dissimilarity matrix, there was a significant difference in the composition (abundance of each sea cucumber species contributing to the overall composition of the animals at each site) between sites ($F_{3,22} = 6.294$, $p = 0.001$). The post-hoc SIMPER tests show that the species contributing the most to the differences between Atata and Kolonga were *H. atra* (50% contribution), *H. hilla* (27%) and *Synaptid* sp. (15%), respectively (**Table 3.2**). The clustering in the NMDS suggests that the abundances of *H. atra* and *H. hilla* were higher at Atata than Kolonga and that the abundance of *Synaptid* sp. was higher at Kolonga than Atata (**Figure 3.3**) (**Table 3.3**).

The species contributing most to the differences between Atata and Sopu were *S. horrens* (36%), *H. atra* (23%) and *H. hilla* (21%) respectively (**Table 3.2**). The clustering in the NMDS plot suggests that *S. horrens* abundance was higher at Sopu than Atata, and that the abundances of *H. atra* and *H. hilla* were higher at Atata than Sopu (**Figure 3.3**) (**Table 3.3**).

The species contributing the most to the difference between Kolonga and Sopu was *S. horrens* (70%) (**Table 3.2**); this is likely because this species was found in relatively high abundances at Sopu whereas none of these individuals were found at Kolonga (**Table 3.3**).

The species contributing most to the difference between Kolonga and Tukatonga was *H. hilla*

(48%) (**Table 3.2**); this is likely because this species was found in relatively high abundances at Tukutonga whereas none of these individuals were found at Kolonga (**Table 3.3**). The main difference in species abundance between Sopu and Tukutonga was attributed to *S. horrens* (**Table 3.2**) which is likely because of its high abundance at Sopu and its relatively low abundance at Tukutonga (**Table 3.3**). However, it is possible that this species' abundance at Tukutonga was underestimated due to them inhabiting the rock habitat. A large abundance of this species was noticed within the band of rocks bordering the seagrass bed as well as within the first few metres from the shore. This area was not covered during sampling. This was because the rocky habitat represented quite a different habitat to where *S. horrens* was found at other sites. However, it is possible that because of this, Tukutonga and Sopu are more similar in terms of *S. horrens* abundance than depicted by this analysis, but that the animals may occupy different habitats at each site.

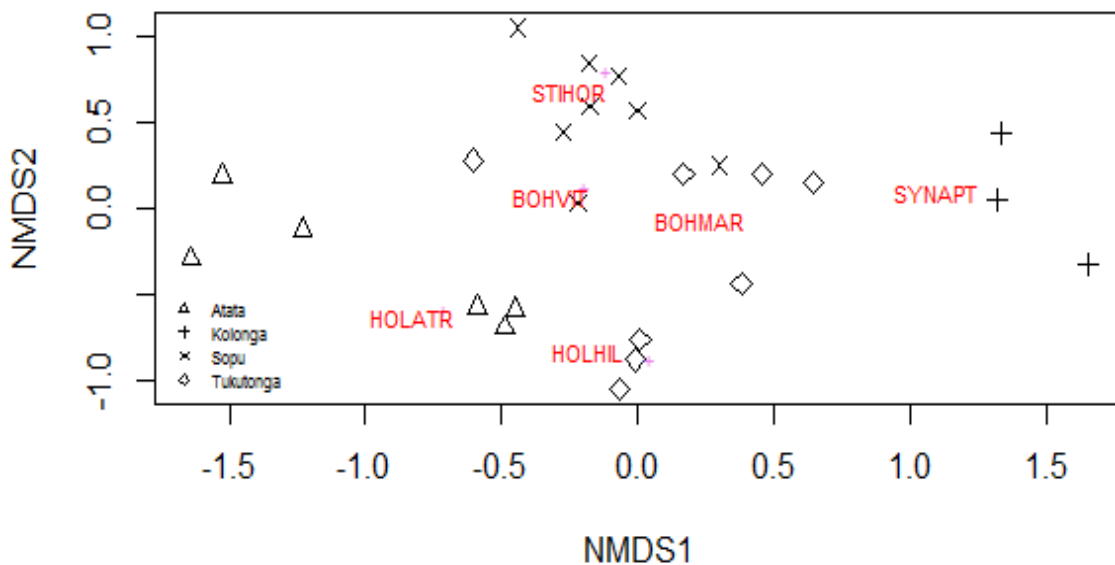


Figure 3.3. Non-Metric Dimensional Scaling (NMDS) plot for sea cucumber diversity dissimilarity (Bray-Curtis) in the seagrass habitat grouped according to site, stress = 0.156. The data points displayed on the graph are representative of the sea cucumber diversity at each site (Δ = Atata, + = Kolonga, \times = Sopu, and \diamond = Tukutonga) for a given sampling event. Species which are influencing the distribution of plots are overlaid on the graph in red font where: HOLATR = *Holothuria atra*, BOHVIT = *Bohadschia vitiensis*, HOLHIL = *Holothuria hilla*, STIHOR = *Stichopus horrens*, BOHMAR = *Bohadschia marmorata*, and SYNAPT = *Synaptid* sp.

Table 3.2. Similarity Percent (SIMPER) matrix for sea cucumber abundance per m².

Site	Species	Cont. (%)	Cumulative (%)
Atata vs. Kolonga	<i>Holothuria atra</i>	49	49
	<i>Holothuria hilla</i>	27	76
	<i>Synaptid</i> sp.	15	91
Atata vs. Sopus	<i>Stichopus horrens</i>	36	42
	<i>Holothuria atra</i>	23	69
	<i>Holothuria hilla</i>	21	93
Atata vs. Tukutonga	<i>Holothuria hilla</i>	35	44
	<i>Holothuria atra</i>	25	75
	<i>Stichopus horrens</i>	11	89
Kolonga vs. Sopus	<i>Stichopus horrens</i>	70	73
	<i>Holothuria hilla</i>	12	86
	<i>Synaptid</i> sp.	8	94
Kolonga vs. Tukutonga	<i>Holothuria hilla</i>	48	55
	<i>Stichopus horrens</i>	18	75
	<i>Synaptid</i> sp.	14	91
Sopus vs. Tukutonga	<i>Stichopus horrens</i>	38	50
	<i>Holothuria hilla</i>	29	87
	<i>Synaptid</i> sp.	4	93

The results display the percent contribution (Cont. %) and the percent cumulative (Cumulative %) contribution of each species to the dissimilarity between sites. The results for the top three contributing species are displayed only.

Table 3.3. Sea cucumber abundance per m² for the seagrass habitat during day sampling events across both sampling seasons 2014 and 2015, and by site and station.

Site/Station	Year	HOLATR	HOLHIL	HOLEDU	STIHOR	HOLLEU	SYNAPT
AT1	2015	0.10	0.03				
AT2	2015	1.13	1.53	0.03	0.3		
SO1	2015		0.08		1.78	0.10	0.03
SO2	2015		0.10		1.0	0.03	0.03
TT1	2015	0.05	4.18		0.03		0.10
TT2	2015	0.08	0.08		0.20		
SO1	2014		0.55		0.45	0.13	
TT1	2014		0.53	0.03		0.03	0.15
AT1	2014	0.30	0.10	0.05			
AT2	2014	2.45	1.28	0.10	0.50		
SO2	2014		0.08		0.80		0.08
TT2	2014	0.05			0.10		0.13
KO1	2014						0.03
KO2	2014						0.13

Kolonga is not included for the year 2015 because no sea cucumbers were sampled in the seagrass during that season. Species codes are the first three letters of the genus name followed by the first three of the species name. Refer to **Figure 3.3** legend for definitions of the abbreviations. HOLEDU = *Holothuria edulis*. For Site/Station, the first two letters define the site (AT = Atata, SO = Sopu, TT = Tukutonga, KO = Kolonga) and the number is the Station identifier (1 = Station one, 2 = Station two).

3. 3. 2. Habitat composition – ‘seagrass’

A PERMANOVA using the Bray-Curtis distance measure and the ‘adonis’ function with 999 permutations specified in Rstudio showed that there was a significant difference in percentage habitat composition per m² between sites around Tonga. The results of the adonis (PERMANOVA equivalent) show a significant difference in habitat composition between sites ($F_{3,26} = 7.167$, $p = 0.001$).

Results of the SIMPER analysis indicates that sand, seagrass and macroalgae are the habitat features contributing most to the differences in habitat composition between sites (**Table 3.4**). These three features were the most dominant features of the ‘seagrass’ habitat that were seen throughout the sample sites around Tonga. The contribution of the habitat variables to differences between sites was relatively low (**Table 3.4**) and are therefore not outlined here because habitat variables are likely to account for relatively small differences in species

abundance between sites. This is also supported by the NMDS plot which displays no significant discernible pattern in the data (**Figure 3.4**).

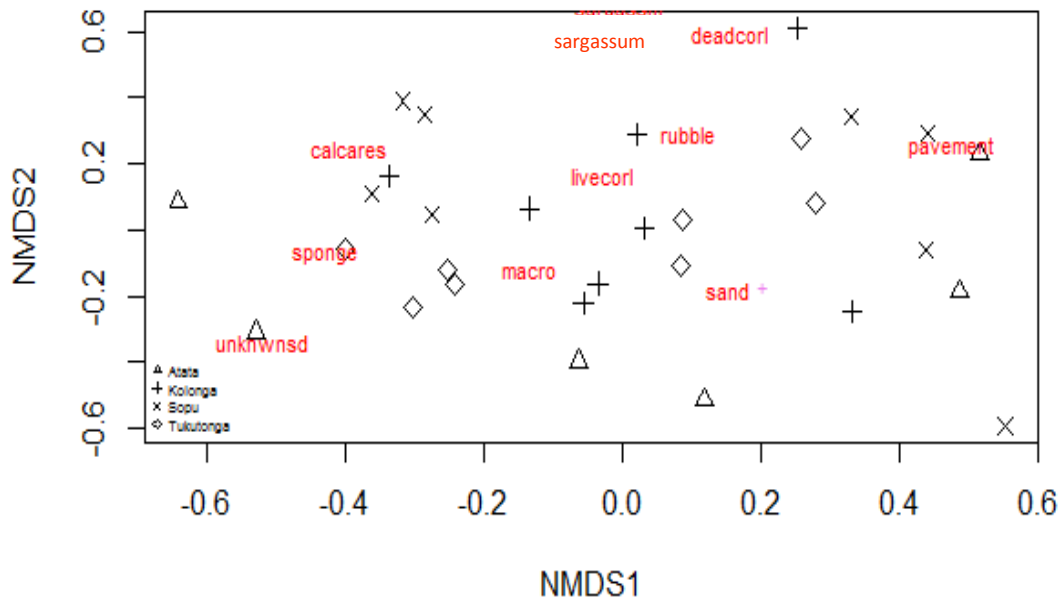


Figure 3.4. Non-Metric Dimensional Scaling plot for percent habitat composition per m² for the 'seagrass' habitat at the four sites; Atata, Kolonga, Sopa and Tukutonga. The data points displayed on the graph are representative of the habitat composition at each site (Δ = Atata, + = Kolonga, \times = Sopa, and \diamond = Tukutonga) for a given sampling event. Habitat variables were: unknwnsd = unknown sediment, calcares = calcareous algae, livecorl = live coral, deadcorl = dead coral, macro = macroalgae.

Table 3.4. SIMPER results (%) dissimilarity Bray-Curtis distance for habitat variables.

Site	Species	Cont. (%)	Cumulative. (%)
Atata vs. Kolonga	Sand	13	34
	Seagrass	11	62
	Macroalgae	7	81
Atata vs. Sopus	Macroalgae	7	25
	Seagrass	6	48
	Sand	5	69
Atata vs. Tukutonga	Seagrass	15	34
	Sand	10	59
	Macroalgae	10	82
Kolonga vs. Sopus	Seagrass	12	35
	Sand	11	67
	Macroalgae	4	80
Kolonga vs. Tukutonga	Macroalgae	7	28
	Seagrass	6	55
	Sand	4	72
Sopus vs. Tukutonga	Seagrass	16	40
	Sand	9	61
	Macroalgae	8	81

Table presenting the top 3 (in order) habitat variables which explained the most variation between sites. Data is per m² and represents the results of 999 permutations.

3. 3. 3. Generalised linear models; effects of habitat variables on *Stichopus horrens* abundance

Generalised linear models were used to determine the effects of habitat composition on the abundance of *S. horrens*. The significant habitat and environmental predictors were light intensity, depth, macroalgae, sand, unknown sediment, pavement and sponge (**Table 3.5**) (refer to **Table 3.1** for descriptions of habitat variables). All of the variables except sponge, which had a small positive effect, had a very small negative effect on *S. horrens* abundance.

Table 3.5. Individual predictor variables of *Stichopus horrens* abundance at Tongatapu (including the offshore island of Atata) in the seagrass habitat as determined by glms with a Poisson distribution; residual df = 1177 for final glm.

Predictor variable	z-value
Temperature	-0.48
Rubble	0.26
Sargassum	-0.02
Padina	-0.02
Live coral	-1.43
Dead coral	1.51
Calcareous algae	-0.27
Soft coral	-0.02
Light intensity	-4.73**
Depth	-6.89**
Macroalgae	-4.67**
Sand	-15.75**
Unknown sediment	-3.22**
Pavement	-2.07*
Sponge	6.082**

Note: ** p < 0.001, *p < 0.05

The glm results indicate that the interactions between three grouped parameters had a significant effect on *S. horrens* abundance within the seagrass habitat (**Table 3.6**). These three groups were: 1. light intensity, unknown sediment and sponge, 2. unknown sediment and sponge, and 3. depth and macroalgae.

Table 3.6. Significant interactions between predictor variables of *S. horrens* abundance in the seagrass habitat around Tongatapu, including the offshore island of Atata, as demonstrated by the glms with a Poisson family distribution; residual df = 1121.

Interaction	z-value
Light intensity × unknown sediment × sponge	2.756*
Unknown sediment × sponge	-2.306*
Depth × macroalgae	3.27*

Note: ** p < 0.001, *p < 0.05

3. 3. 4. Differences between time of day, stations, and years – *Stichopus horrens*

Stichopus horrens was not found at Kolonga at any sampling event. Thus, this site was excluded from analysis. Due to the use of multiple tests, the Bonferroni correction was applied to the following datasets to adjust for this. This resulted in an adjusted significance level of 0.003, owing to performing 13 statistical tests. At AT2, there was no significant difference in the mean ranks of *S. horrens* abundance between 2014 and 2015 ($H = 0.001$, $df = 1$, $p = 0.976$) (**Table 3.7**). The difference between Stations 1 and 2 was not analysed as either raw or transformed data, because *S. horrens* was not present at Station 1, but was present at Station 2.

At SO1, the differences in *S. horrens* abundance between years could not be analysed because no transformations could normalise or homogenise the data (**Figure 3.5**). There was a statistically significant difference in *S. horrens* abundance between stations in 2015 ($H_1 = 19.63$ $p < 0.001$) (**Table 3.7**). There was no significant difference between years at Station 2 ($H_1 = 1.179$, $p = 0.278$) or between stations in 2014 ($H_1 = 2.29$, $p = 0.130$) in *S. horrens* abundance (**Figure 3.5**).

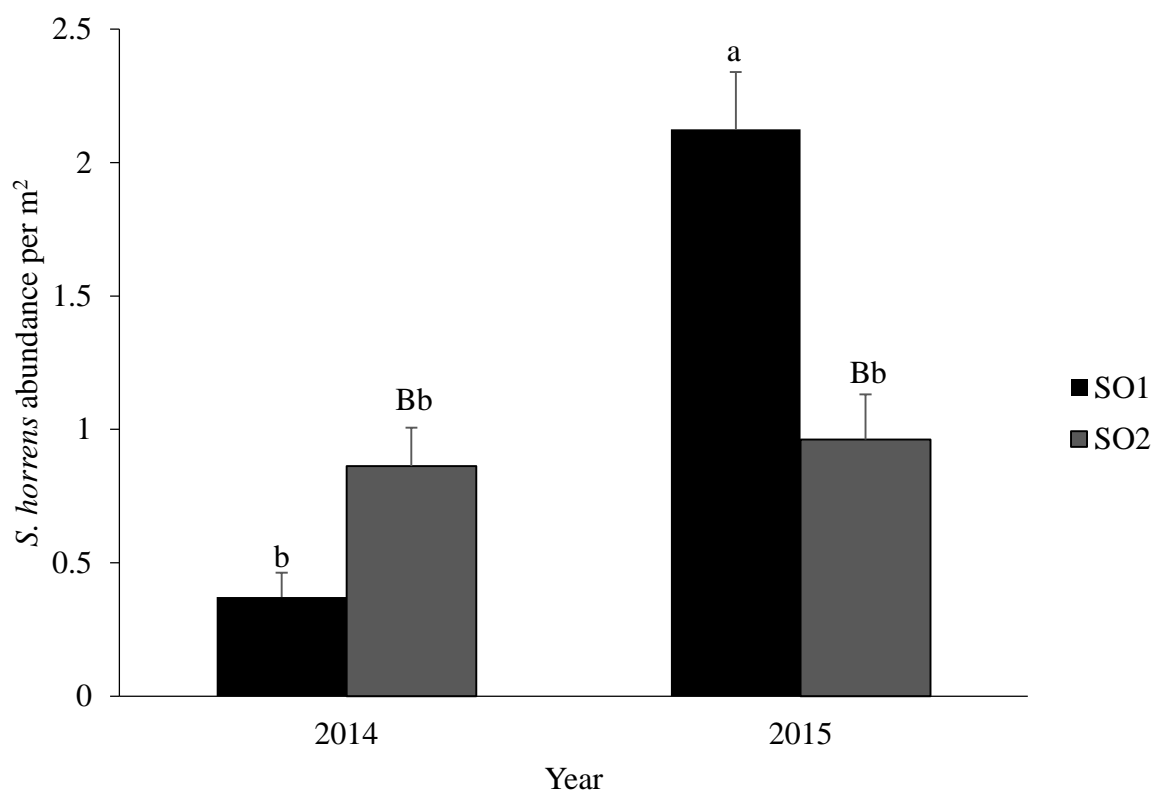


Figure 3.5. Average *S. horrens* abundance per m² (+ standard errors) in the seagrass habitat at Sopo reef on the island of Tongatapu displayed by year and Station (Sopo Station 1, SO1; Sopo Station 2, SO2). Data for SO2 and for both Stations in 2014 was square root transformed for analysis but raw data is presented here. Bars sharing lowercase letters within a year were not statistically significantly different from each other, and bars sharing uppercase letters between years were not significantly different from one another.

TT1 2014 data was not analysed because no *S. horrens* were sampled in the quadrats, however it should be noted however that *S. horrens* was present at this site. During a sampling event it was noticed that this species was present closer to the shore in the rocks which was a habitat which was not sampled. There was no significant difference between stations in 2015 ($H = 0.583$, $df = 1$, $p = 0.445$) or between years ($H = 0.029$, $df = 1$, $p = 0.866$) (Table 3.7)(Figure 3.6).

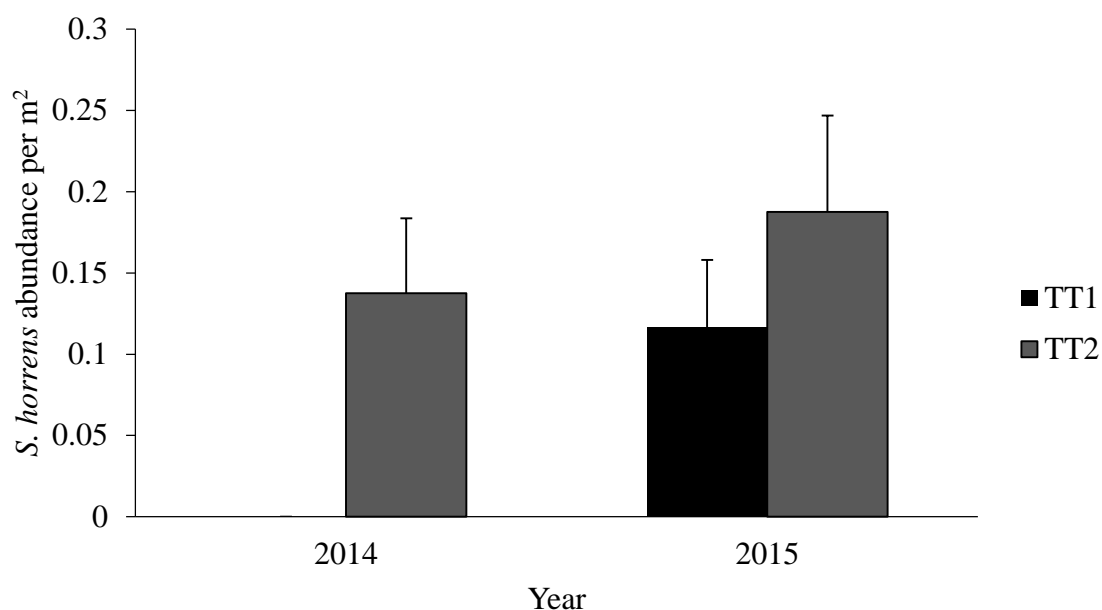


Figure 3.6. Average *S. horrens* abundance per m² (+ standard error) in the seagrass habitat at Tukutonga on the island of Tongatapu by year and station. TT1 2014 is not included here because there were no individuals sampled in the seagrass habitat at that sampling event.

Table 3.7. Summary of Kruskal-Wallis test results where H = Kruskal-Wallis test statistic, n/a = no data transformation was performed, year = testing for a significant difference between years, time of day = testing for a significant difference between day and night, station = testing for a significant difference between stations. For all tests, df = 1.

Analysis	Transformation	H stat
AT2: year	Square root	0.001
SO1 2014: time of day	n/a	0.923
SO1 2015: time of day	n/a	1.695
SO2 2014: time of day	n/a	0.011
SO2 2015: time of day	n/a	0.505
SO 2015: Station	Square root	19.63**
SO 2014: Station	Square root	0.029
SO2: year	Square root	1.179
TT2 2014: time of day	Square root	0.178
TT2 2015: time of day	n/a	0.197
TT1 2015: time of day	n/a	2.031
TT 2015: Station	n/a	0.583
TT: year	n/a	0.029

Note: ** p < 0.001, *p < 0.05

3.3.5. Species richness

A one-way ANOVA showed a significant difference in species richness between sites ($F_{stat_{3,26}} = 17.17$, $p < 0.001$) (**Figure 3.7**). There was, however significantly higher species richness at Atata, Sopu, and Tukutonga than at Kolonga ($p < 0.001$) (**Figure 3.7**).

A nested ANOVA showed that there was no significant effect of time of day within site on species richness ($F_{stat} = 0.899$, $df = 4, 22$, $p = 0.481$). However, the identity of the species did differ. Chalkfish (*Bohadschia marmorata*), for example, was only recorded in night samples. There was no significant difference in species evenness between sites ($F_{stat} = 1.058$, $df = 2$, $p = 0.368$) as determined by a one-way ANOVA on square root-transformed data.

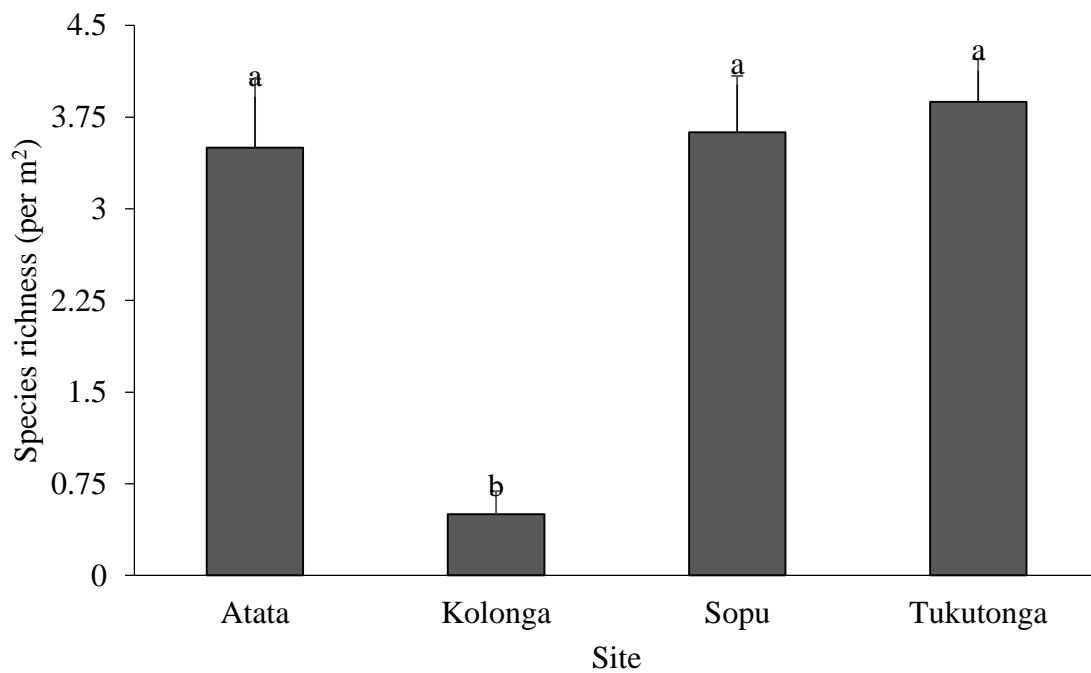


Figure 3.7. Average species richness per m² (+ standard error) for the seagrass habitat of each site sampled in Tongatapu island group; Atata (n = 6), Kolonga (n = 8), Sopu (n = 8), Tukutonga (n = 8). The average species richness is the average of the number of species found at each sampling event. Bars sharing letters are not statistically different from each other as determined by an ANOVA and post-hoc Tukey test.

3. 3. 6. Species diversity; Shannon-Wiener index

There was only one species recorded at any time at Kolonga in the seagrass habitat which was either *Synaptid* sp. or *Bohadschia marmorata*. Therefore, the site of Kolonga was left out of the following analysis but it should be noted that when it is included in the dataset, the relationship between site and diversity does not meet the assumption of homogeneity of variances indicating that Kolonga represents an outlier ($K = \text{Inf}$, $df = 3$, $p < 2.2 * 10^{-16}$). When Kolonga was excluded, there was no significant difference in species diversity between sites ($F_{\text{stat}} = 0.297$, $df = 2, 19$, $p = 0.746$) (**Figure 3.8**).

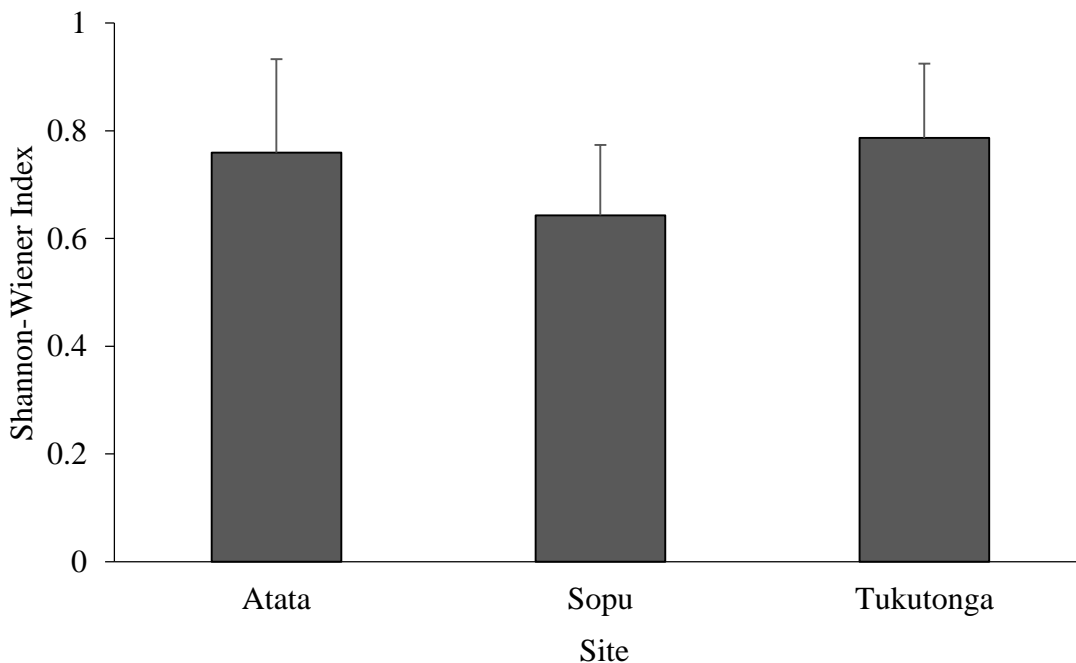


Figure 3.8. Average Shannon-Wiener Index (+ standard errors) for the seagrass habitat at each site around Tongatapu. The data presented here are for all sampling events per site including both 2014 and 2015 data. Kolonga was left out because there was never more than one species present at this site per sampling event.

3. 3. 7. Juvenile *Holothuria leucospilota* aggregation – Atata Station 2

A two sample t-test revealed no significant difference in the abundance of juvenile *H. leucospilota* between A2N14 and A2D14 (AT2 night sample 2014, and AT2 day sample 2014, respectively) ($t_{\text{Stat}} = -0.16$, $df = 14$, $p = 0.873$) (**Figure 3.9**). There was no night sampling of AT2 in 2015, thus, the comparison between the abundance of juveniles between years was performed on day time data only to avoid an unbalanced design. The difference in

juvenile abundance between years was almost significant ($t_{Stat} = 2.09$, $df = 9$, $p = 0.066$) (**Figure 3.9**). The quantities of juveniles were counted per 0.25 m^2 and it was these data that were analysed to avoid extrapolating abundances to 1 m^2 which may not necessarily have been accurate.

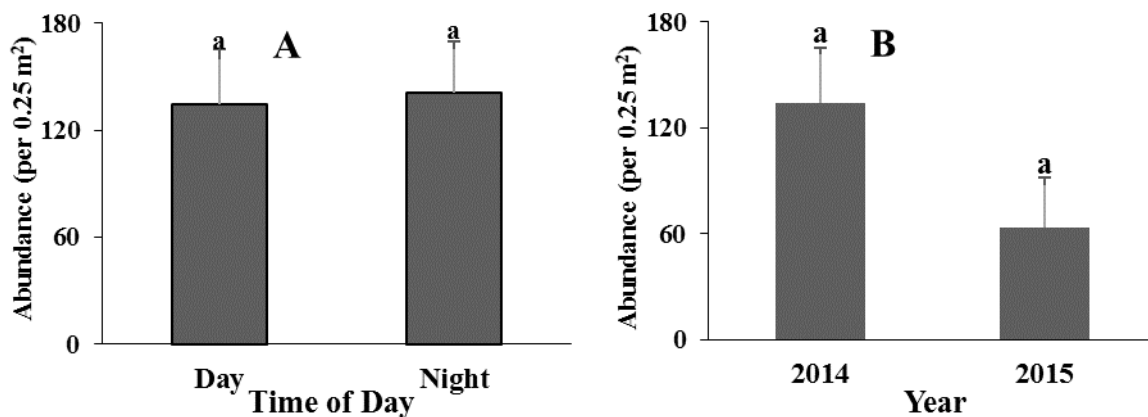


Figure 3.9. The abundance of juvenile *Holothuria leucospilota* per 0.25 m^2 counted at Atata Station 2 in the years 2014 and 2015. Panel A is displaying data for night and day sampling events in the 2014 sample season only. Panel B is displaying day time sampling data for both sampling seasons (2014 and 2015). Bars sharing letters are not statistically significantly different from each other as determined by two-sample t-tests.

3. 3. 8. Length-frequency

Holothuria atra

There were no *H. atra* at SO1 or SO2 in either 2014 or 2015, so this site was not included in the following length frequency graphs (**Figure 3.10**). The most common length range of *H. atra* in both years lay between 10 – 15 cm, and the highest abundance of *H. atra* was at the offshore island of Atata (**Figure 3.10**).

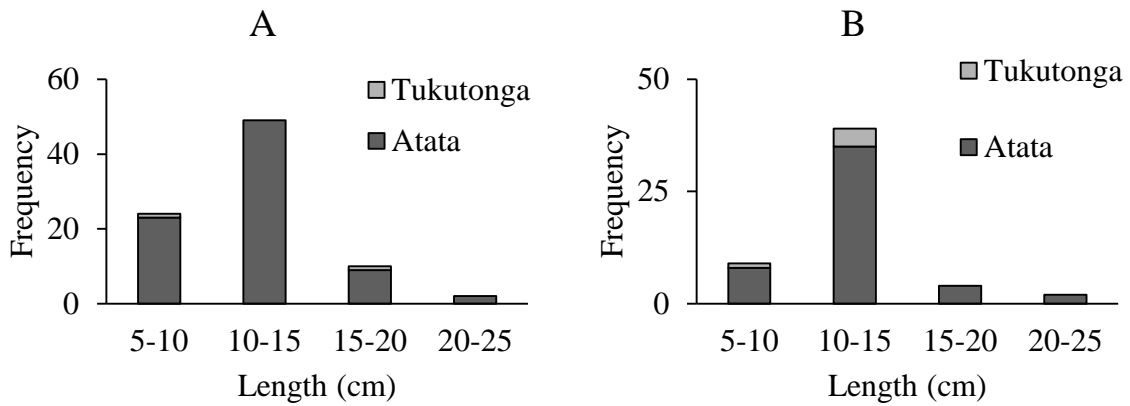


Figure 3.10. Length-frequency graphs for *Holothuria atra* sampled in 2014 (A) and 2015 (B) at Tukutonga and Atata.

Holothuria hilla

In 2014, the highest length-frequency range was between 5 – 10 cm, while in 2015 the highest was 10 – 15 cm (**Figure 3.11**). The two sites in 2014 with the highest abundance of *H. hilla* were Sopu and Atata, while in 2015 the two sites with the highest abundance were Atata and Tukutonga (**Figure 3.11**).

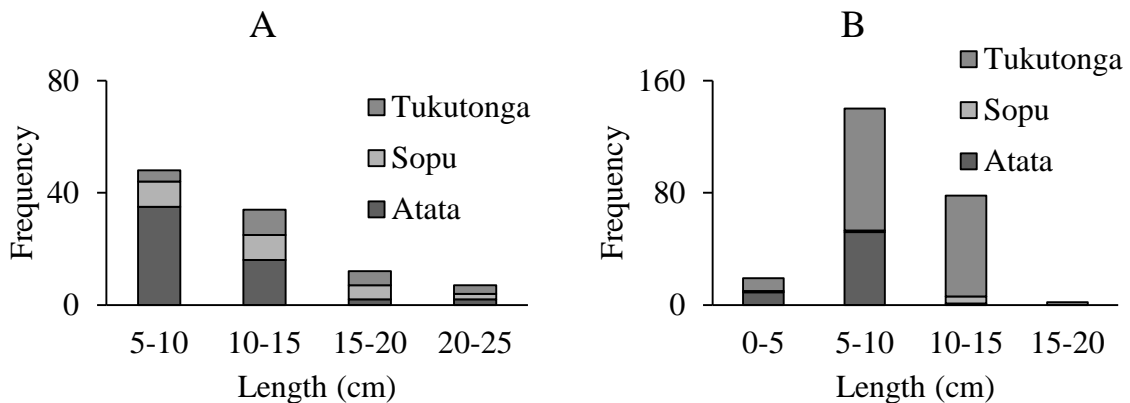


Figure 3.11. Length-frequency graphs for 2014 (A) and 2015 (B) for *Holothuria hilla* sampled at Tukutonga, Atata, and Sopu.

Holothuria edulis

H. edulis was only found once during the sampling season of 2015. This was found at A2D15 and had a length of 10 cm. In 2014, the highest abundance of *H. edulis* was found at Atata. Regardless of site, the length range for *H. edulis* fell between 5 and 20 cm (**Figure 3.12**).

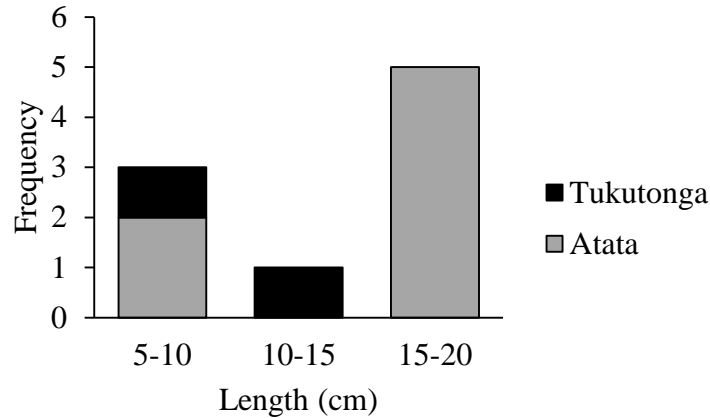


Figure 3.12. Length-frequency graph for *Holothuria edulis* in the 2014 sample season at Tukatonga and Atata.

Stichopus horrens

In both sampling seasons (2014 and 2015), the highest frequency of *S. horrens* was found at Sopu (**Figure 3.13**). The two most common size groups across both years fell between 5 – 15 cm in length (**Figure 3.13**).

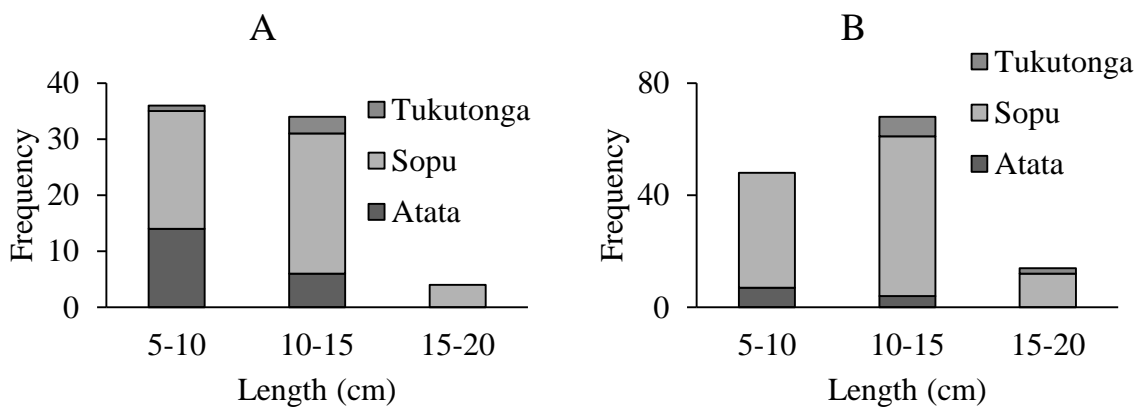


Figure 3.13. Length-frequency graphs for 2014 (A) and 2015 (B) for *Stichopus horrens* at Tukatonga, Sopu, and Atata.

Holothuria leucospilota

In both sampling seasons, Sopu was the only site from which adult *H. leucospilota* was found (**Figure 3.14**). For S1N14 (SO1 night sample in the year 2014) the length data was not collected for this species, however, the total number of this species across all 8 transects in the sargassum habitat was 37 while only one was found in the seagrass.

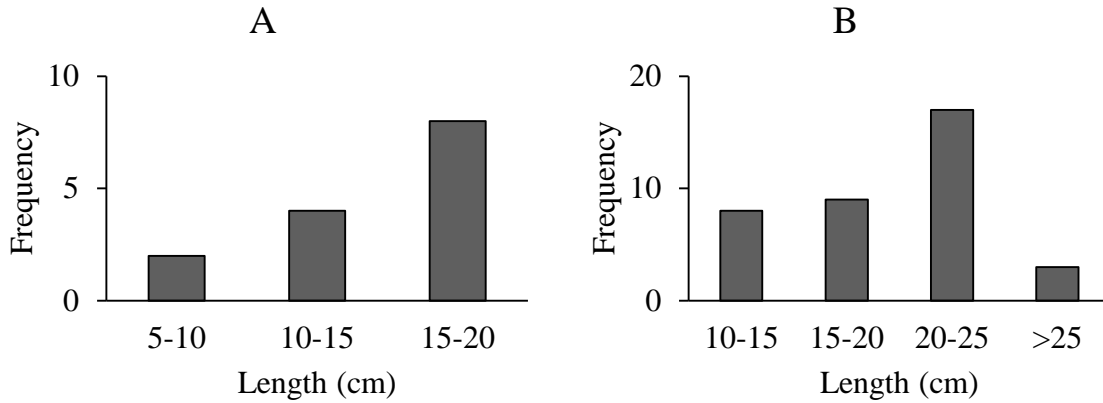


Figure 3.14. Length-frequency graphs for 2014 (A) and 2015 (B) for *Holothuria leucospilota* at Sopa only.

Bohadschia marmorata

Only 4 individuals of this species were encountered in the 2015 field season and all of them were at Kolonga. Seventeen individuals of this species were counted in the 2014 field season. This species was typically only observed during night sampling, and was virtually undetectable during the day except when raking through sediments disturbed a buried individual. In 2014, Kolonga and Tuketonga were the two sites with the highest number of *B. marmorata*, and the majority of animals fell between the size range of 5 and 15 cm (**Figure 3.15**).

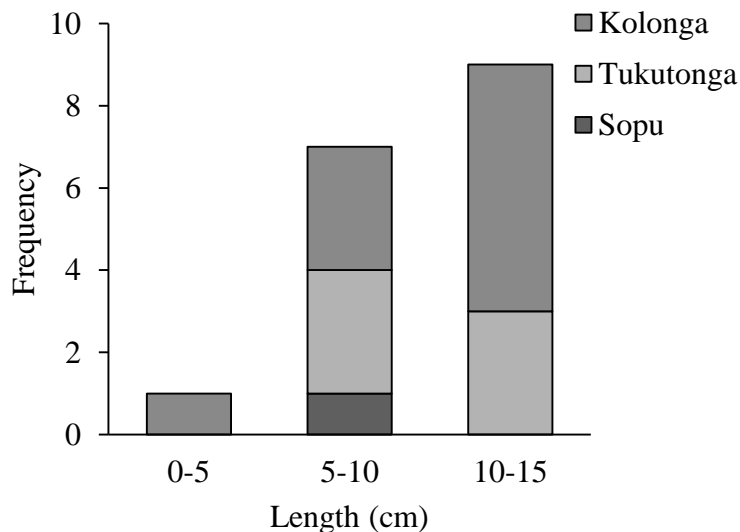


Figure 3.15. Length frequency graph for 2014 for *Bohadschia marmorata*.

Synaptid sp.

Length data were not collected for the *Synaptid* sp. because this very long species (> 1 m) has the capacity to contract or expand its length considerably. Thus measures of length would not have been consistent. Therefore, only abundance of *Synaptid* sp. is presented (**Figure 3.16**).

This species was only found at the onshore sites of Tuketonga, Kolonga and Sopu but not found at the offshore island of Atata. The highest abundance of *Synaptid* sp. was at Kolonga in the 2014 sampling season, and the year 2014 generally demonstrated the highest abundance of *Synaptid* sp.

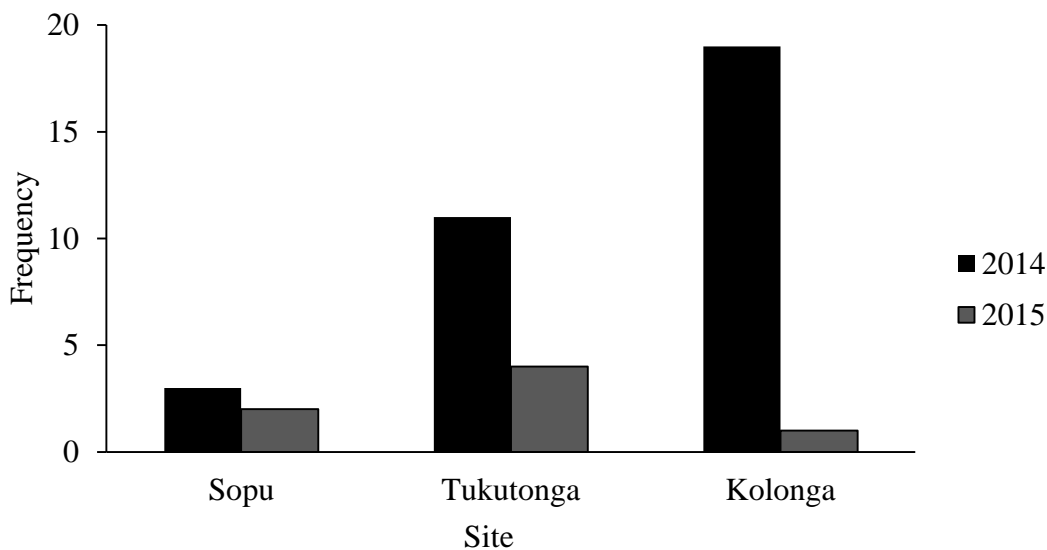


Figure 3.16. 2015 and 2014 abundance for Sopu, Tuketonga, and Kolonga, for the sea cucumber species identified as *Synaptid* sp.

3. 4. Discussion

The abundance of *Stichopus horrens* ranged from 0 to 2.5 per m² and this species' abundance was variable between sites and Stations. *Stichopus horrens* was only found in the seagrass habitat during sampling periods, but was also seen on rocky substrates which did not encompass the sampling habitat. The lowest abundance and diversity of all species was consistently seen at Kolonga (**Figure 3.7 & Figure 3.8**) (**Table 3.3**).

3. 4. 1. Differences in sea cucumber composition between sites

In the present study, there was a significant difference in sea cucumber abundance between sites. The defining habitat variables that distinguished sites from one another were sand, seagrass and macroalgae. However, because the differences in these three variables contributed so little to the differences between sites (See Section 3. 3. 2.), it is unlikely that the difference in sea cucumber abundances between sites is due to the environmental and habitat variables measured in this study. However, this cannot be entirely ruled out, because sea cucumber species may occupy slightly different niches within a habitat to prevent competition. This has been demonstrated by sea cucumbers that occupy the same microhabitat, but which use slightly different feeding methods, allowing them to coexist (Jangoux, 1980). The small habitat differences between sites presented here may be enough to accommodate different species, and abundances of species if different microhabitats and resources were available. However, it is more likely that the major differences between sites in sea cucumber diversity and richness is due to fishing pressure, environmental health, or hydrographic conditions not measured.

Kolonga had lower species richness and diversity than Sopu, Tukatonga and Atata. It is possible that this was due to the location and local conditions of Kolonga, but it is also possible that this was due to fishing intensity. Kolonga was the most exposed site sampled, and is one of the most exposed and low-lying sections of Tongatapu, where coastal erosion has taken place consistently over the last 20 or so years (Climate Resilience Sector Project, 2012). A low-lying road running parallel to the shoreline offers the coastline in this area some protection, however, Kolonga village, which is situated less than 2 m above sea level, has flooded during storms in the past (Climate Resilience Sector Project, 2012). Given that the majority of households use septic tanks (which have been known to overflow at times) and houses on the village periphery use pit latrines, it is possible that flooding results in nutrient exchange between land and sea (Climate Resilience Sector Project, 2012). The area of seagrass directly in front of the village is covered in a brown film, which is most likely algae resulting from nutrient loading (pers. obs. November 2014 and May – July 2015). Additionally, epiphytes have been reported on seagrasses at Kolonga (and were also observed during this study) which can be indicative of nutrient loading (McGlathery, 2001; Climate Resilience Sector Project, 2012). Furthermore, the sediment at Kolonga was anoxic in appearance and odour (pers. obs. Kolonga 2014 and 2015). Sea cucumbers play an important role in aerating sediments through bioturbation, and they can also control eutrophication

through grazing on algae (Worm et al., 2000; Purcell, 2004). Thus, the low sea cucumber abundance observed at Kolonga is particularly concerning because the role of sea cucumbers in buffering systems against eutrophication is reduced, and thus the ecosystem may suffer as a result. Critically, when a system becomes prevalent in micro- and macroalgae, it can reduce seagrass cover and have significant impacts on biodiversity (McGlathery, 2001).

Low sea cucumber abundance at Kolonga has been prevalent since at least 2012, when ecological surveys showed three sea cucumber species were present but all in low abundance (Climate Resilience Sector Project, 2012). These findings are compatible with the results presented in this study, except only two species of sea cucumber were observed in the seagrass and rubble/sargassum habitats at Kolonga; *Synaptid* sp. and *Bohadschia marmorata*. Thus, it is likely that for at least 3 years, sea cucumber abundance and richness at Kolonga has been low, indicating that stocks have been unable to recover from fishing efforts. The Climate Resilience Sector Project (2012) reports that high local community input into commercial fishing activities saw the harvest of inter-tidal and reef-dwelling sea cucumbers. Furthermore, subsistence fisheries exist in the area year-round for the species *Stichopus horrens*, *Holothuria atra*, *Holothuria coluber*, and *Bohadschia marmorata*. Of these species, the only one which was encountered during field sampling in the present study either during 2014 or 2015 was *B. marmorata* and even then that was only present at relatively low abundance. It is possible that there is higher fishing pressure at Kolonga (see below) and this may have resulted in the low richness and diversity of sea cucumbers seen at Kolonga, as opposed to the relatively exposed nature of the site.

The low richness and diversity of sea cucumbers at Kolonga is concerning, not only because it appears that population numbers have been unable to recover in the last three years, but also because sparse numbers can present major problems for reproduction. Sea cucumbers are broadcast spawners, casting their gametes into the water where fertilisation occurs externally (McEuen, 1988). When the density of sea cucumbers is low (i.e., in overfished areas) the probability of gametes meeting is also low, which has a negative effect on reproductive success (Bell et al., 2008). This can lead to the Allee effect, where low densities of individuals become limiting in terms of population growth, and can eventually lead to the extinction of populations (Courchamp et al., 1999). This is when the effectiveness of closed seasons and size restrictions are called into question, because population size, density, and reproduction are now the factors limiting population recovery (Bell et al., 2008). In this instance, it is recruitment from the metapopulation that becomes an important means by

which to replenish populations (Lipcius et al., 2008). Bell et al. (2008) suggested that restocking no-take zones with hatchery and wild-caught sea cucumbers, as well as rearing wild animals in sea pens until they reach sexual maturity, could be viable ways to restock exploited populations. It is likely that a situation requiring such action is being demonstrated in Kolonga, where sea cucumber populations are so sparse that they are unable to recover.

Kolonga is located in a rural area relatively far from the main centre of Nuku'alofa. It is possible that there is more intense fishing pressure in Kolonga compared to Tukumotonga and Sopo because of its relative isolation, and potentially fewer alternative income earning opportunities. By comparison, Atata is an offshore island that supports one village and a resort, with community-based marine resource management in the form of a designated Special Management Area (SMA) and Fish Habitat Reserve (FHR). An SMA gives the community authority to manage how its inshore resources are used, and can provide the community with the means to more effectively manage the marine environment (Gillett, 2009). At Atata, the area of the SMA is 745 hectares, and the area of the FHR is 178 hectares (**Figure 3.17**) (Tonga Fisheries Division, 2010). General conditions of the SMA include: only registered people and fishing vessels are allowed to fish there, all other people must apply for a fishing permit from the coastal community management committees (CCMCs), destructive fishing methods are prohibited and marine animals cannot be harvested for the aquarium trade (Tonga Fisheries Division, 2010). In addition, Atata has some of its own fishing conditions, including that curryfish species cannot be gutted at shore (Tonga Fisheries Division, 2010).

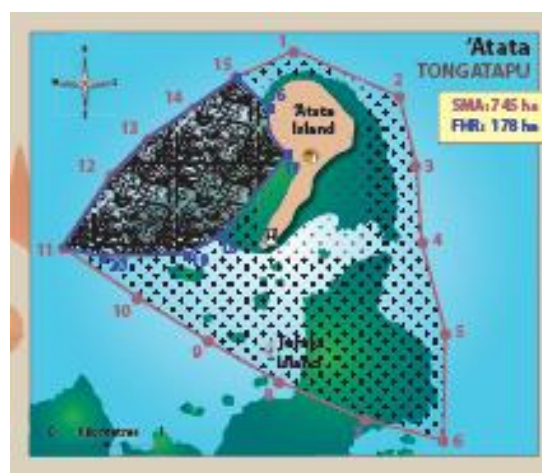


Figure 3.17. Image taken from SMA brochure (2010) prepared by the Tonga Fisheries Division, showing the Special Management Area (SMA) and the Fish Habitat Reserves (FHR) at the offshore island of Atata.

Atata, like Kolonga, is also isolated and opportunities for employment may be fewer compared with Tukatonga and Sopu. However, it appears that the community-based marine regulations at Atata have been successful at preventing sea cucumber overfishing as observed at Kolonga. With such strict regulations, higher abundance of commercially fished species of higher monetary value were observed at Atata than at the other sites (**Table 3.3**). Clustering in the NMDS suggests that the abundance of *H. atra* was highest at Atata compared to the three other sites (**Figure 3.3**). At Atata, the most prevalent species sampled were *H. atra*, *S. horrens*, *H. leucospilota* (juveniles), *H. edulis* and *H. hilla*, while *B. marmorata* was only counted once (most likely because this species is rare in seagrass habitats and the seagrass was the only habitat sampled here). Of these species, all are sought after for commercial fishery export, subsistence fishing, or for the aquarium trade (Kinch et al., 2008, Purcell et al., 2010). However, *H. atra* and *H. edulis* are the species reported as being sold in Tonga for commercial export (Conand et al., 2013; Purcell et al., 2014). Thus the higher abundance of *H. atra* at Atata compared to Tukatonga, Sopu and Kolonga, and the prevalence of *H. edulis* at Atata compared with the other sites, is likely reflecting the success of the management regimes in place.

Synaptid sp. was noticeably absent from Atata, and relatively common at all three of the onshore sites. It is possible that the higher abundance of commercial species such as *H. atra* and *H. edulis* may be preventing the *Synaptid* sp. from establishing at Atata through competition. According to Gause's principle of competitive exclusion 'complete competitors cannot coexist' (Hardin, 1960). It is widely accepted that closely-related species are also similar in their ecological niches, however, the extent to which they are similar may differ between groups of species (Losos, 2008). Resource partitioning and niche differentiation between closely related species may be mechanisms by which competitive exclusion can be avoided. Substratum partitioning between sea cucumber species has been demonstrated in multiple ecosystems, and may be related to differences in tentacle surface texture between species (Roberts, 1979). However, the capacity of holothurians to selectively feed and their ability to partition resources based on particle size has been contested by Hammond (1982). Further evidence supporting resource partitioning in abundant sea cucumbers is presented by Sloan (1979), who showed that abundant species occupied different microhabitats by utilising different food sources and altering their feeding behaviour. *Synaptid* sp. feed on algae and organic matter and generally prefers a combination of seagrass and sand habitats or reef flats (James, 2001; Conand et al., 2010; Lampe, 2013). The other species at Atata fed primarily on

sediment (pers. obs. Tonga 2014), and thus it is unlikely that these species were in competition with one another, based on their food sources alone.

An alternative explanation for the lack of *Synaptid* sp. at Atata lies in the large number of juvenile *Holothuria leucospilota* specimens counted at AT2 in the present study (**Figure 3.9**). It is possible that larvae of the *Synaptid* sp. were unable to establish at this site because of the *H. leucospilota* juvenile population observed. However, this is based on the assumptions that the juveniles of both species would occupy the same ecological niche, that the *H. leucospilota* population established first, and that priority effects are in evidence.

A priority effect is the effect that early colonising species have on the establishment of future colonising species (Symons & Arnott, 2014). It is possible that the relatively high abundance of juvenile *H. leucospilota* and the relatively high species richness seen at Atata (compared with Kolonga) may be influencing the ‘invasibility’ of this community to *Synaptid* sp. through competition. Additionally, establishment can also be influenced by the predators present at the establishment site, as demonstrated by settlement patterns in *P. californicus* (Cameron & Fankboner, 1989). Thus, competition and predation could be two factors influencing the establishment of *Synaptid* sp. at Atata. However, it is also possible that *Synaptid* sp never made it to Atata through barriers to dispersal.

Sea cucumber species, including *Holothuria scabra*, *Synapta digitata* and *Parastichopus californicus*, have a larval phase (Grave, 1903; Cameron & Fankboner, 1989; Bell et al., 2008). Larval phases have a high dispersal capacity, but this is not to say that there are not barriers to their successful dispersal (Borrero-Pérez et al., 2011). Barriers to the dispersal of marine species can include; geographic distance, low survival rate of larvae, and physical barriers such as water channels (Hellberg et al., 2002; Pérez-Losada et al., 2007). The distance between Tongatapu mainland and Atata is approximately 10 km, with Egeria channel located between the two. The reef between Tongatapu mainland and Atata island is relatively well connected (**Figure 3.18**) and given that prevailing current between Tongatapu and Atata is from a north easterly direction (pers. obs. May 2015 to July 2015), it is not unlikely that currents would be able to transport *Synaptid* larvae in the direction of Atata, even if dispersal was stochastic along the reef. It has been suggested that hydrogeography plays a role in determining larval dispersion capacity in the sea cucumber species *Holothuria mammata* (Borrero-Pérez et al., 2011). Additionally, it has been shown in *Holothuria nobilis* that genetic differences can exist between populations occupying different regions, but that

isolation within regions is unlikely (Uthicke & Benzie, 2001). A similar pattern has been demonstrated between neighbouring subpopulations of the sea cucumber species *Cucumaria frondosa*, which displayed relatively high genetic connectivity (So et al., 2011). In summary, it appears unlikely that isolation by distance is the mechanism causing the lack of *Synaptid* species in the areas sampled on Atata Island. However, it is possible that this species is able to disperse to Atata, but that it has not established there due to barriers to establishment such as resource partitioning and competitive exclusion.

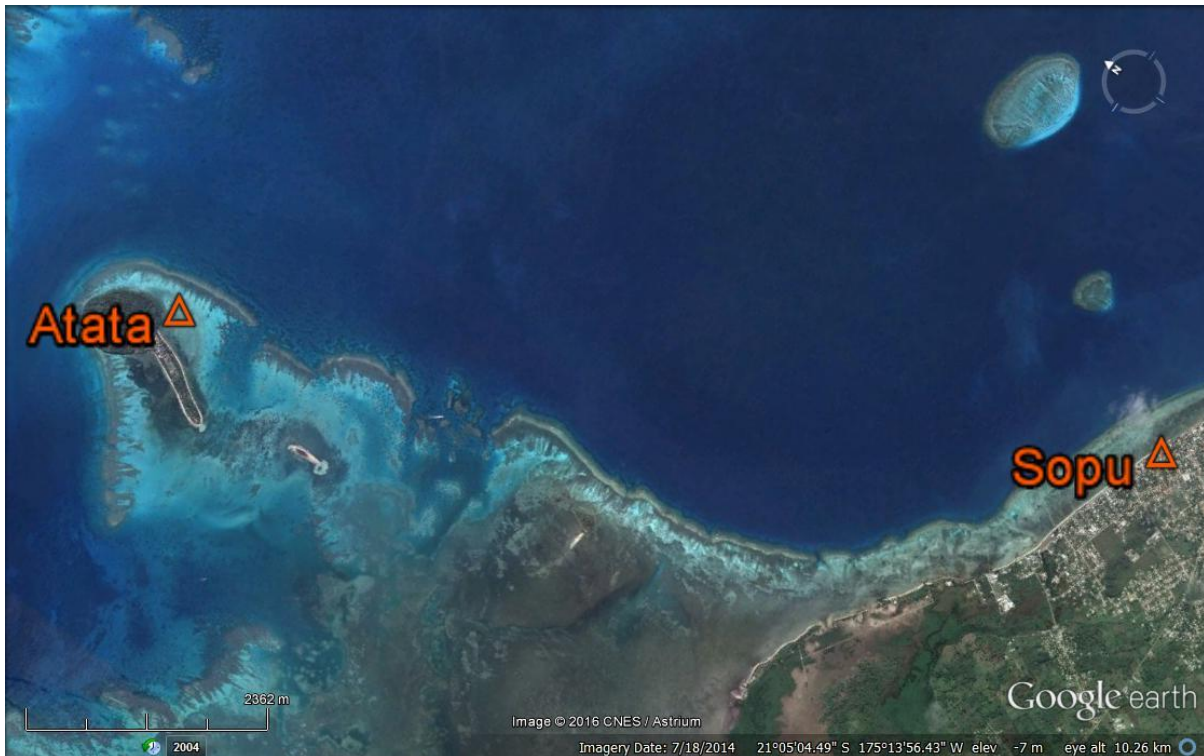


Figure 3.18. Image from Google Earth showing the reef between the mainland of Tongatapu and the offshore island of Atata. Reef patches displayed here are the ridges present in the water off the side of the mainland, punctuated by shallower sandy flats (blue/white in colour) and deeper ocean (dark blue).

The lack of *Synaptid* sp. at the offshore island of Atata compared with the onshore sites of Tukatonga, Sopu and Kolonga, is unlikely to be due to the unsuitability of the habitats sampled, given that the habitats were a combination of seagrass and sand, relatively sheltered and with access to reef flats (unsampled habitat) further offshore. It is possible that *Synaptid* sp. was not able to successfully establish at the sites sampled at Atata, possibly due to barriers to dispersal from the mainland combined with the relatively dense and diverse species assemblages encountered at Atata which may have prevented establishment of *Synaptid* once it arrived. Additionally, different levels of predation, and different predators may have been

in abundance at Atata compared to the onshore sites, combined with different wind and wave action. The effects of different hydrological processes may be greater between stations at Atata than at other sites, which may also influence the dispersal and establishment of *Synaptid* sp.

Within Atata, the highest diversity and species richness was seen at Station 2 (leeward side of the island). Although for the purpose of the statistical analyses the stations were grouped (to attain a more general context of Atata as a whole as opposed to individual areas), this phenomenon was still in evidence, and the difference between stations in terms of community assemblage was more prevalent at Atata than any of the other sites (pers. obs. Tonga 2014 and 2015). Wave action and wind exposure have been demonstrated as two factors affecting holothuroid abundance, with windward assemblages being less diverse than leeward assemblages (Kerr et al., 1993; Drumm et al., 2011). It is possible that the prevailing wind direction is affecting the assemblages at the two stations on Atata Island.

3. 4. 2. *Stichopus horrens* ecology

Stichopus horrens has been described as ‘diurnally cryptic’; hiding beneath rocks during the day and inhabiting the middle reef flat which was partially submersed at low tide (Kerr et al., 1993; Eriksson, 2006). It has been found living on reef flats and upper slopes, but generally on hard substrates such as rubble and rock (Kinch et al., 2008; Drumm et al., 2011). It has also been observed on macroalgae, at the base of seagrasses and in soft benthos habitats (Eriksson, 2006). In this study, searches for *S. horrens* were conducted by searching extensively and systematically through the seagrass, and at Sopu and Atata this species occurred in sandy/seagrass habitats. At Tukatonga, few specimens were found in the seagrass, while more tended to inhabit the rocks. Specimens were also found inhabiting rocks at Sopu (**Figure 3.19**), but seemed to be less abundant in the rock habitat than in the seagrass, although this was not quantified. Thus it appears that *S. horrens* is able to inhabit both rocky/rubble habitats and seagrass/sand habitats. However, it is unclear why *S. horrens* might be more abundant in the seagrass at Sopu and in the rocks at Tukatonga. It is possible that similar factors which may be contributing to *Synaptid* sp. distribution may also be affecting the difference in habitat choice of *S. horrens* between Sopu and Tukatonga.



Figure 3.19. Photograph of *Stichopus horrens* taken at Sopu reef in the sample season 2014 inhabiting the rocks bordering the shoreline at low tide.

The abundance of *S. horrens* appears to be slightly negatively affected by the individual habitat variables of light intensity, depth, macroalgae, sand, unknown sediment and pavement (See Section 3.3.3.). The results of the present study showed that as light intensity increased, *S. horrens* abundance decreased. This would be the expected finding for a cryptic, nocturnal species. Many other species of sea cucumber such as *Actinopyga agassizii*, *Stichopus monotuberculatus*, and *Actinopyga lecanora* show an aversion to light, taking shelter under rocks or in crevices during the day time (Purcell et al., 2012). Some authors consider this behaviour to be an anti-predator tactic (Nelson & Vance, 1979; Francour, 1997; Mercier et al., 1999; Dance et al., 2003). An increase in sand cover and pavement means that there is a reduction in potential hiding places, thus the negative effect of light intensity, sand cover and pavement on *S. horrens* abundance is likely a reflection of its cryptic behaviour and is possibly due to predator evasion tactics. Given that light intensity decreases as depth increases, it is interesting that in the present study, an increase in depth resulted in a decrease in *S. horrens* abundance.

A negative effect of depth on abundance has also been demonstrated in *Stichopus variegatus*, a shallow-water species that is restricted to the coastline (Conand, 1993a). In the species *Isostichopus badiionotus*, distribution was unrelated to depth but more related to shelter from wave action (Sloan & von Bodungen, 1980). Coastal areas also have nutrient inputs from the terrestrial system (Voss et al., 2011), and thus nutrients and depth (in some instances) in terms of sea cucumber distribution may be interrelated. It is possible that these factors are influencing the depth distribution of *S. horrens*. It is interesting that *S. horrens* abundance decreased with increasing macroalgal cover given that macroalgae could provide cover for this species. However, macroalgae can sometimes be an indication of eutrophication, and can result in oxygen depletion within a local environment (McGlathery, 2001). It is possible that this may be influencing the aversion of *S. horrens* to high macroalgal abundance.

Furthermore, macroalgae do not stabilise sediment as effectively as seagrass which can increase turbidity in macroalgal dominant areas (McGlathery, 2001). Thus, the effects that macroalgae have on the marine system may not be suitable for *S. horrens*. It is interesting that while macroalgae has a negative effect on *S. horrens* abundance, the presence of sponges had a positive effect.

It is possible that the presence of sponges provided additional habitat complexity which may be attractive to sea cucumbers. Higher habitat complexity has been related to post-settlement survival in the Atlantic cod (*Gadus morhua*), by acting as predatory shelter (Lindholm et al., 2001). Furthermore, sponges can act as a habitat for the recruitment of juvenile species, act as a home for microbes, and can remove inorganic nitrogen from the system (Duckworth et al., 2007; Hoffmann et al., 2009). It is possible that sponges in the seagrass system construct a favourable microhabitat for *S. horrens* via their role in the environment, thus resulting in the positive relationship seen in the present study.

3. 4. 3. *Stichopus horrens* abundance between stations, years and sites

There were significantly higher numbers of *S. horrens* at Sopus in 2015 compared with 2014. The sample season for 2015 occurred in May – July, while the sample season for 2014 occurred during November. At SO2, there was no significant difference in *S. horrens* abundance between years, suggesting that at SO1 some external factor was influencing this change. There have been two separate studies which have demonstrated different results in terms of the sexual reproductive patterns in *S. horrens*. Hu et al. (2013) demonstrated that this species follows a lunar reproductive cycle, while Muthiga and Conand (2014) demonstrated

an annual reproductive cycle (January – March) for *S. horrens*. An annual reproductive cycle has been demonstrated in other species such as *Actinopyga mauritiana* and *Parastichopus californicus* which reach their sexually reproductive phase in warmer months (Cameron & Fankboner, 1986; Hopper et al., 1998). However, in accordance with the findings of Hu et al. (2013), Toral-Granda (2008) also suggested that *S. horrens* may reproduce year-round.

Year-round sexual reproduction has been demonstrated in other species of sea cucumber such as *Isostichopus badionotus*, *Holothuria mexicana*, and *Holothuria scabra*, for which sexual reproduction occurs throughout the year, albeit with some time periods being more active than others (Guzmán et al., 2003; Ramofafia et al., 2003). Aggregations of individuals during or prior to reproductive periods is a phenomenon demonstrated by some sea cucumber species (McEuen, 1988). *H. scabra* was demonstrated to reproduce throughout the year (Ramofafia et al., 2003) but also formed aggregations prior to reproduction, which appeared to follow a lunar cycle (Mercier et al., 2000a). The sea cucumber *Cucumaria miniata* demonstrated a clumped distribution during the reproductive season, with neighbours often spawning synchronously with one another (Sewell & Levitan, 1992). It is possible that the increase in *S. horrens* abundance at SO1 in 2015 compared to 2014 may be the result of aggregations prior to lunar spawning, recruitment from other areas, or a higher rate of asexual or sexual reproduction at SO1 compared to SO2 (See Section 2.4.4.). It is also possible that predation rates or disturbance differed between the stations and this may have affected juvenile survival rate and thus the population numbers. *S. horrens* were absent from TT1 in 2014 but present in 2015, it is possible that this is also related to reproduction. Thus, more information about the sexually reproductive behaviour of *S. horrens* is required to determine whether changes in abundance may be related to aggregation behaviour or reproduction.

3.4.4. Length-frequency of sea cucumbers around Tongatapu

Stichopus horrens was found in the highest abundances at Sopu, and in the size range of 10-15 cm. It is possible that high traditional fishing pressures in this area are elevating the rate of asexual reproduction (See Section 2.4.4.) leading the population to be comprised of a relatively large number of small individuals compared with other areas. The size at sexual maturity for *S. horrens* is thought to be 25 cm (Muthiga et al., 2010) or 245 g (Yacinthe in Friedman et al., 2008c). Thus it appears that the individuals of *S. horrens* are too small in any of the study areas to sexually reproduce. Few large, sexually mature specimens of *S. horrens* have been found in the Philippines indicating high fishing pressure and reduced reproductive

capacity compared to the relatively high population numbers (i.e. the size of sea cucumbers were small but there were many of them) (Olavides et al., 2010). This study suggests that the high population numbers (of relatively small individuals with a median length of between 10 and 15 cm) might be the product of recruitment whereby numbers are maintained from a source population outside of the sample area (Olavides et al., 2010). It is possible that something similar is being seen in *S. horrens* populations in the areas sampled in the present study, and that the sexual reproductive capacity of these animals may be limited. In Queensland, a minimum size limit of 17 cm is in place for *S. horrens* (Bruckner, 2006), and in Tonga, the minimum size limit is 12 cm (wet) for the taking of this species (Government of Tonga, 2008). It appears that to ensure this species can continue to sexually reproduce size limits may be need to be raised, possibly for both artisanal and commercial activities.

The most common length-frequency group for the species *Holothuria atra* was 10-15 cm, which is encouraging given that the length at first sexual maturity has been reported as 16.5 cm in New Caledonia, and 19 cm in Fiji (Conand, 1993b; Seeto, 1994). For *Holothuria hilla*, the most common length-frequency was 5-10 cm and the size of males at sexual maturity is thought to be 7.6 cm (Mamhot, 2013), which indicates that these animals likely fall within the size scope for sexual reproduction. The high abundance of *H. hilla* in 2015 may be due to increased rates of asexual reproduction by fission during cooler months (Lee et al., 2008a). *Holothuria edulis* was sampled in relatively low abundances, and was found only at Atata and Tukutonga. The habitats sampled in the present study are consistent with the seagrass, sand and inner reef flat habitats described as this species' preference (Dissanayake & Stefansson, 2012; Conand et al., 2013). Thus, the likely reason for their relatively low abundance is exploitation by fishing, because they are a harvested species in this region (Conand et al., 2013). *Bohadschia marmorata* has a size at sexual maturity of 90 g and a maximum length of approximately 26 cm and an average length of 18 cm (Purcell et al., 2012). Thus, the sizes reported for the specimens in the current study were likely below those required for sexual reproduction. The abundance of *Synaptid* sp. were consistently higher in the 2014 sample than in the 2015 season. Given that this species is not commercially fished, it is likely that this is related to environmental variables.

Adult *Holothuria leucospilota* showed a definite preference for the rubble/back-reef habitat where it was found with its posterior end underneath rocks and with its anterior end protruding and tentacles actively feeding (pers. obs. Sopu, Tongatapu 2014 and 2015). This has also been demonstrated in other studies (Jangoux, 1980; James, 2001; Kinch et al., 2008).

Previous studies have shown that *H. leucospilota* favours rubble/rock habitat (Romero & Cabansag, 2014). Adult *H. leucospilota* were sampled only at Sopu in rubble/rock habitats, and Kolonga was the only other site at which the rubble/back-reef was sampled in this study, thus, the absence of *H. leucospilota* in this seemingly suitable habitat is possibly due to fishing pressure in this area. Size at sexual maturity for this species has been described as 180 g (Purcell et al., 2012), 21.8 cm for females and 25.2 cm for males (Mamhot, 2013). There are some individuals counted in the present study which fell within these size ranges, and it appears that more specimens of greater size were abundant in the 2015 season, but the reasons for this are unclear. At the offshore island of Atata, juvenile specimens of *H. leucospilota* were found in relatively high density aggregations at Station 2 in the seagrass beds. It appears that this species begins its juvenile life in the seagrass habitat and then migrates further offshore to live its adult life in the rubble/back-reef habitat.

In summary, it appears that the sea cucumber populations at Kolonga are too low to repopulate and there is urgent need of further surveying, and possibly restocking with hatchery-reared sea cucumbers or those from the metapopulation. The findings of this study also indicate that resource partitioning, physical barriers, hydrogeography and competition may play a role in species establishment and dispersal. Furthermore, *Stichopus horrens*, an important subsistence and commercial fishery species in Tonga, may be at risk of inhibited sexual reproductive rates due to the majority of sampled specimens being below the sizes reported in the literature for sexual maturity. However, this should be verified with more intensive sampling. Furthermore, in species which are capable of asexual reproduction, population levels are likely to be maintained by this mechanism, but the impacts of continuous asexual reproduction and lowered sexual reproduction rates on genetic structure and population resilience should be investigated further. Additionally, habitat preferences and variables for *S. horrens* indicates that this species may prefer areas with complex habitats, thus highlighting the importance of protecting and conserving seagrass beds in the Pacific. This may mean addressing problems such as nutrient loading to prevent micro and macroalgal blooms and human and animal traffic (such as pigs) in the nearshore environment.

4. Trace metal accumulation in sediment and holothuroid tissues in Tongatapu

4. 1. Introduction

Tonga is classed as a small island developing state (United Nations, 2016), and pollution control is often less rigorous in developing countries than developed countries (Nriagu & Pacyna, 1988). Given the strong reliance the Tongan people have on nearshore fisheries as a food source, monitoring trace metals in coastal ecosystems is very important. Metal contaminants can enter the aquatic environment via dumping, effluent and leaks, terrestrial runoff, irrigation, and erosion, among many others (Förstner & Wittmann, 2012). They can also enter the aquatic environment through atmospheric refuse burning, dust, biogenic sources (such as vegetation), fossil fuel burning, construction, and electricity generation (Nriagu & Pacyna, 1988; Nriagu, 1989; Senesil et al., 1999; Callender, 2003; Gholampour et al., 2016). Many of these potential sources of metal contamination are present in the Tongatapu island group.

Tongatapu is home to approximately 70% of the Tongan population, and in recent years has undergone rapid urbanisation (Kaitani & McMurray, 2006). In addition, there are approximately 2,000 farmers on Tongatapu, although unlike many other countries, farming is mainly crop-based instead of animal-based (Völkel, 2010; Havea, 2014). Historically, farming in Tongatapu has been subsistence-based, but this is now changing. Cash-cropping, particularly for squash which is highly prized on the Japanese market, is more prevalent, and this is associated with an increase in fertiliser and chemical use (Fakava 2000; Tupou, 2000; Dutton & Helu, 2005). Traditionally, long fallow periods have allowed natural soil fertilisation, however over the last several decades, fallow periods have shortened and artificial fertilisers have become more prominent to allow more intense farming (Völkel, 2010).

The use of fertilisers and pesticides in terrestrial systems can represent a significant source of trace metals in the aquatic environment resulting from water-based chemical run-off (Guzmán & Jiménez, 1992; McDowell, 2010). In Tonga, fertilisers and pesticides are misused and often applied too liberally (Massey University, 2015). In this region, the fertilisers used in squash farming contain Cd, Pb, and Hg, and it is thought that these, in combination with pesticide use, are placing a trace metal load on Tongan agricultural soils (Clothier et al., 2005). Despite the trend towards organic farming in Tonga (Havea, 2014), it is possible that the misuse and overuse of fertilisers may be a source of trace metals in this environment.

Historically, no specific treatment or disposal process for contaminants such as thermometers containing Hg or hospital wastewater has occurred, thus the contamination of ground or wastewater with trace metals is possible (Picardi, 2002). In addition, the open dump in Tongatapu did not have groundwater, run-off or air contamination controls in place (Picardi, 2002). It is unclear if the circumstances around healthcare and wastewater waste have changed since the work published by Picardi (2002).

The dumping of ship waste represents a significant source of pollution in the marine environment (Islam & Tanaka, 2004; Butt, 2007), especially given that the regulation of ocean dumping from shipping vessels in Tonga, has been reported as 'ineffective' (SPREP, 2014). The main port in Tonga is located on Tongatapu, in the capital city of Nuku'alofa, which is subject to heavy ship traffic. This therefore represents a significant possible source of marine pollution to the Kingdom of Tonga. In addition to shipping waste and vessels as a source of marine contaminants, wastewater and sewage leakage also represent sources of trace metals in the environment in this region. In 2008, there was no wastewater system in Tonga, with individual households being in charge of their own wastewater disposal, often leading to marine and groundwater pollution due to water disposal in open soaked pits (Newton, 2008). Septic tanks are commonly used around Tonga, and the sewage taken from such tanks is disposed of at the Tapuia landfill (neighbouring Fanga'uta lagoon). It has been noted that the size of the landfill is not large enough to cope with the quantities of sewage from tanks (Newton, 2008). Additionally, the design and maintenance of septic tanks is often poor, resulting in groundwater contamination (Newton, 2008). Human waste is not the only sewage waste of concern, and with each Tongan household owning between 3 and 14 pigs, animal waste may also act as a source of pollutants (Newton, 2008). Thus there a multitude of

potential sources of trace metals in the marine environment due to the inputs of human sewage, ocean waste disposal from ships, and wastewater/groundwater contamination.

In addition to water-based inputs of trace metals, the atmosphere can also be a trace metal source. Tonga Power Limited is the sole electricity producer in Tonga, with greater than 20,000 customers collectively across the island group (Tonga Power Limited, 2015). Ninety-two percent of energy production at this company comes from diesel generation (Tonga Power Limited, 2015). Fossil fuel combustion can release metallic compounds into the atmosphere (Vouk & Piver, 1983), thus the reliance on fossil fuels for energy in Tonga could be acting as a source of trace metals in the environment.

Not only does fossil fuel burning result in metal release into the atmosphere, but refuse burning also achieves the same end. Traditionally, the waste in Tonga has been biodegradable, but as plastics, foils and other non-biodegradable resources have been imported, they have also been treated in the same way as biodegradable rubbish – by burning or discarding in swampy areas (Dutton & Helu, 2005). It is thought that 10,000 tons of municipal solid waste is generated in Tongatapu each year, one third of which is burned or dumped, with some of rubbish dumping occurring in the ocean and on beaches (Asian Development Bank, 2014; SPREP, 2014). In addition, airport quarantine and port waste has historically been burned outside following the technical failure of incinerators (McDowall & Drummond, 2006). Thus, there is potential for the atmosphere in Tonga to act as a source of atmospheric trace metals due to refuse and fossil fuel burning.

Although there are significant potential sources of trace metals in the Tongan environment, there have been steps towards using renewable energy, organic farming, and controlling the amount of solid waste being dumped and burned by the Tongan people. However, there are significant health effects associated with human exposure to trace metals above safe concentrations, as well as biological impacts of elevated metal concentrations on aquatic biota.

Although trace metals are essential and normal components of living things, when concentrations of these metals become high enough, they can be toxic to the organism (Bryan, 1971). The biological impacts of elevated metal concentrations on aquatic biota include the disruption of homeostatic processes, inhibition of embryonic development, and DNA damage (Viarengo, 1994; Bolognesi et al., 1999; King & Riddle, 2001). In addition, trace metals have been shown to negatively influence survival and growth in juveniles of the

sea cucumber *Apostichopus japonicus* (JunFeng et al., 2014). Other studies on sea cucumbers have quantified the concentrations of trace metals in body tissues, and generally demonstrated (due to the lack of correlation between sediment and tissue metal concentrations), that these bioindicator potential of these organisms may be questionable (Xing & Chia, 1997; Denton et al., 2009; Givianrad et al., 2014).

Concentrations of trace elements in sediments and Holothuroid tissues were measured to determine if trace metals were accumulating in coastal ecosystems in the Kingdom of Tonga.

4. 2. Methods

4. 2. 1. Sediment and sea cucumber collection; The Kingdom of Tonga

Sea cucumbers and sediments were collected from the same sites, and at the same stations within each site as outlined in Section 3. 2. 1. (Table 4.1). Sediment were collected in 2014 and 2015 (with the exception of Atata which was sampled in 2014 only due to logistical constraints), with sediment samples taken from two stations at each site. Sea cucumbers were sampled in 2014 only, and were collected from the same stations and sites as the sediment samples. Up to eight sea cucumber species were collected at each site including; *Holothuria hilla*, *Bohadschia marmorata*, *Holothuria atra*, *Stichopus chloronotus*, *Bohadschia vitiensis*, *Holothuria edulis*, *Holothuria leucospilota*, and *Stichopus horrens*.

Table 4.1. Description of sampling sites from which samples were collected in Tonga.

Site	Description	No. sediment	No. sea cucumber
Atata	Offshore island ~ 30-minute boat ride from Tongatapu	4	6
Kolonga	Onshore rural site located near Kolonga village on the North-East side of the island	8	8
Sopu	Onshore site located in Nuku'alofa capital	8	6
Tukutonga	Onshore site located in Nuku'alofa capital	8	7

The animals were collected at both high and low tide from all sites within the same week. Following collection, the animals were immediately put in a bucket of seawater collected from the same site. The sea cucumbers were transferred into sealed plastic bags and frozen at

-18 °C until they could be dissected. Nitrile gloves were worn to avoid cross-contamination. Dissections occurred within a week of animal collection. Animals were weighed and dissected by making a vertical cut from the cloaca to the mouth. The body fluid was drained and each internal organ was bagged separately to avoid contamination between organs. A section of body wall with muscle bands attached was also sampled. The tissues sampled were the body wall, respiratory tree, intestine and gonads (when present, as not all specimens had gonadal tissue). The intestine here refers to the entire digestive tract, and no distinction was made between sections. Dissection instruments were rinsed in sea water and wiped clean with Kim-wipes between sites to prevent inter-site contamination, and nitrile gloves were worn and regularly changed. Once dissected, the tissues were frozen, again at -18 °C.

Sediment samples were collected at the same sites as the sea cucumbers. As described in Section 3.2.1., there were two stations at each site. From each of these stations, a sediment sample was collected from both nearshore (seagrass) and offshore (rubble/sargassum habitat or the very outskirts of the seagrass). Two stations at each site were sampled as a trade-off between resource availability and the prevention of sample bias. At Atata and Tukatonga, no rubble/sargassum habitat could be reached due to it having a steeper shore depth gradient relative to the others. At Tukatonga, an outer seagrass sample was taken in 2014, but this location could not be reached in 2015 due to a higher tidal height at the time of collection. Therefore, a nearshore seagrass and mid-shore seagrass sample was taken. In total, four samples of approximately 500 g wet weight were taken from an area of approximately 2 m² at each site by scooping the top 5 cm sediment layer into a plastic jar or a sealable plastic bag depending on resource availability. Nitrile gloves were worn while handling sediments. The top 5 cm layer of sediment was taken because this encompasses both the top 5 mm layer on which sea cucumbers feed (Dar & Ahmad, 2006), and other sediments which may be circulated in rough weather conditions or by wave action. The lids of each jar were sealed shut using parafilm to prevent leakage and were frozen at -18 °C. Both sediment and tissue samples were then transported to New Zealand in a cool-box, and were in transition for approximately 12 hours before they were placed in a -20°C freezer in a PC2 facility at the University of Canterbury.

4. 2. 2. Sample analysis

4. 2. 2. 1. Sediment metals

Sediment samples of an approximate dry weight of 100 g were oven-dried at 30°C to avoid loss of Hg. Dried sediments were sieved to < 2 mm in a closed bag in a sterilised fume hood using clean nitrile gloves to avoid contamination. The sample was then poured into a sealed plastic bag and stored at room temperature. Approximately 1 (\pm 0.004) g of each sediment sample was weighed into individual polycarbonate centrifuge tubes. Ten ml of 20% ultrapure hydrochloric acid was added in 2 ml increments using an air-displacement pipette to minimise carbon dioxide production from digestion of calcium carbonate. Once the samples had settled, 4 ml of ultrapure 50 % nitric acid was added to each sample and they were capped and left for 24 hours. The samples were digested in a heating block at 85°C for one hour. Once digested, they were removed from the hot plate and allowed to cool overnight before being made up to 20 ml with Milli-Q water. Samples were moved to a metal-free clean-room to be prepared for analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). For preparation, an air-displacement pipette was used to measure 0.5 ml of each sample into tubes specifically for ICP-MS use. Ten ml of a solution with the following constituents: 2% nitric acid (HNO₃), 0.5% hydrochloric acid (HCl) and 0.1% L-cysteine (aqua-regia/L-cysteine solution), was then added to each of the samples. The addition of L-cysteine was to stabilise the Hg. The samples were inverted 17 times to ensure they were thoroughly mixed, and were then submitted for ICP-MS analysis.

4. 2. 2. 2. Sea cucumber tissues

Due to the small size of the tissue samples, many required the use of small tubes for acidification and digestion. Prior to use, all tubes used for samples <100 mg dry weight (tissues) were acid-washed (10% HNO₃ acid) for 48 hours, rinsed three times in Milli-Q water and allowed to air dry for 48 hours in a metal-free clean room. At all times, gloves, a hair cover, a lab coat and shoe covers were worn to minimise any contamination. The larger tubes that were used for tissue samples > 100 mg and for all sediment samples did not require acid washing as previous research has shown that they were suitable for use without acid washing.

All tissues were thawed, rinsed in filtered seawater remove any excess sediment or fluid, blotted dry and placed into Eppendorf tubes. Each tube was left without the lid on, and covered in nappy liner (thermal bonded non woven) to allow any water to escape during the

freeze-drying process. Once dry, tissues were frozen in a zip-lock bag. Once removed from the freezer, the tissues were left to reach room temperature with the zip-lock bag still closed, to prevent any condensation forming. The samples were then capped, and if they were to be digested within 24 hours they were left at room temperature, but any longer they were stored at -20°C .

The procedure for the analysis of tissue trace metals was adapted from Gaw et al. (2012), with the addition of cysteine to the final solution. For chemical analysis, the tissues were divided into two sizes – large (> 100 mg dry weight) and small (< 100 mg dry weight). For large tissues 1 ml ultrapure HNO_3 and 0.2 ml ultrapure HCl , and for the small tissues 0.35 ml ultrapure HNO_3 and 0.07 ml ultrapure HCl , were added using an air displacement pipette. The acid volume used for the small tissues was approximately one third of the acid volume used for the large samples to maintain proportion. The samples were left for 24 hours, and then heated to 85°C . The large samples were placed in a heating block, and the small samples were placed on a hot plate to heat for one hour. They were left to cool at room temperature for a further 24 hours. The cooled samples were made up to 10 ml and 5.4 ml for the ‘large’ and ‘small’ tissues respectively with a solution containing 2% HNO_3 /0.5% HCl /0.1% L-cysteine solution. After being made up to volume, the samples were allowed to stand for a further 24 hours before being transferred to a metal-free clean room where a $3 \times$ dilution was performed by adding 1 ml of sample to 2 ml 2% HNO_3 /0.5% HCl /0.1% L-Cysteine solution. These samples were inverted 17 times to ensure they were completely mixed. The samples were then submitted for ICP-MS analysis.

Initially, to determine which tissue types to analyse, all tissues (intestines, body wall, longitudinal muscle bands, gonads (where available), and respiratory tree) from 6 animals of the species *Stichopus horrens* were analysed for trace metals. Based on these results, which showed that the muscle bands and body wall had similar patterns and concentrations of metal uptake while the other tissues showed quite varying concentrations of metals, the decision was made to analyse all tissues except the muscle bands (**Figure 7.1**). The body wall was chosen over the muscle bands because of the common consumption of the dried body wall as Beche-de-mer throughout Asia (Barron et al., 2014).

An Agilent 7500cx ICP-MS with 2-260 AMU quadrupole mass analyser, discrete dynode detector (9 orders of magnitude), and autosampler ASX-500 fitted with a collision cell (He gas) for the removal of polyatomic interference was used for this work. The elements

analysed included: ^{57}Fe , ^{59}Co , ^{60}Ni , ^{63}Cu , ^{66}Zn , ^{75}As , ^{111}Cd , ^{201}Hg , and ^{208}Pb (sum of ^{206}Pb , ^{207}Pb , and ^{208}Pb). Rhodium (^{108}Rh) was used as an internal standard and added on-line.

4. 2. 2. 3. Quality control

A blank was included for every ten samples to measure background concentrations of trace elements. Additionally, duplicates of sediment samples were also added as a quality control, but duplicates of tissue samples were not included because of the relatively limited number of tissues and their small size. The means and standard deviations were calculated for the blanks and if background concentrations accounted for $\geq 10\%$ of the sample metal concentration ($\mu\text{g l}^{-1}$) the value of the blank was subtracted from the sample. Certified Reference Materials (CRMs) were also included in the analyses to assess the accuracy of the analytical techniques used. For each batch of sediment samples (2014 and 2015 analyses), a measured dry weight (approximately 1 g each) of two CRMs of the Standard Reference Material (SRM) 2702 for measuring “Inorganics in Marine Sediment” were used. SRM 2976 mussel tissue ($n = 5$) and SRM 1577c bovine liver ($n = 3$) were used to validate the sea cucumber tissue analyses and to determine their reproducibility. The recoveries are reported in the Appendix (Section 7.1) and were deemed acceptable.

4. 2. 3. Data analysis

The data from ICP-MS are given as metal concentration in $\mu\text{g l}^{-1}$. The detection limits for each element were determined as the measured value of the first standard which fell within 15% of the specified value (Table 4.2).

Table 4.2. Calculated detection limits ($\mu\text{g g}^{-1}$) for the elements (and isotopes) analysed by ICP-MS.

Element	2014 sediment	2015 sediment	Tissues (initial)	Tissues (secondary)
^{57}Fe	4.0	0.4	10.6	3.7
^{59}Co	0.04	0.04	1.1	0.4
^{60}Ni	0.04	0.04	1.1	0.4
^{63}Cu	0.04	0.04	1.1	0.4
^{66}Zn	0.4	4.1	10.6	37.2
^{75}As	0.04	0.04	1.1	0.4
^{111}Cd	0.04	0.04	1.1	0.4
^{201}Hg	0.4	0.4	10.6	3.7
^{208}Pb	0.04	0.04	1.1	3.7

The ‘Tissues (initial batch)’ column refers to the detection limits for the first run of tissues on the ICP-MS, and the ‘Tissues (secondary batch)’ column refers to the detection limits for the second run of tissues on the ICP-MS.

For a given metal, if 50% of the sediment or tissue metal concentrations were above the detection limit then they were statistically analysed. If they were analysed and any samples were below the detection limit (BDL) then they were assigned a value of half of the detection limit. All data was \log_{10} transformed for analysis and no outlier analysis was conducted. Data was considered significant if $p < 0.05$.

For the statistical analysis of the sediment data, all data was \log_{10} transformed for analysis before a one-way Analysis of Variance (ANOVA), followed by a Tukey post-hoc test, was conducted to determine significant differences between years, sites, and distance from shore. For the analysis of years and distance from shore, a one-way ANOVA was used because it yields the same results as a two-sample t-test and it met the requirements for the statistical testing conducted here. For the analysis of between sites, only the nearshore data (for sediment) was tested because at Tukutonga and Atata there was not a balanced number of far and near samples, which may have confounded the results. For the tissue analysis, due to low n-values between species and in order to prevent testing (statistically) across different species, habitats (distance from shore) and sites, the only species which could be statistically analysed for differences between sites and between tissues was *Stichopus horrens*. The data was \log_{10} transformed and two-factor ANOVAs with a post-hoc Tukey test were used to determine the differences between sites, tissues and the interaction between the two variables. No outliers were removed from any data set, because each data point represents 'real' data.

The results obtained in this study were in dry weights. Some of the regulatory values for tissue metal concentrations reported in the literature were in wet weight, thus the average moisture content of the tissues in the present study was calculated, and the values in the present study were converted to wet weight.

Linear regression was used to determine if there was a significant relationship between sediment and tissue metal concentrations and to assess the possibility of using sea cucumbers as sediment metal indicators. Because there were multiple sea cucumber samples collected from each station within each site, the average concentrations for each specific tissue type (body wall, intestines, gonads (where applicable) and the respiratory trees) were calculated, and paired with the appropriate sediment metal reading, then used and treated as an absolute value in the regression analysis. For example, if a sediment sample was taken from Atata Station 2 in the nearshore, then all of the metal values for each tissue across all of the species sampled from that area were averaged. It is this average that was used in the regression

models. A linear regression to explore the relationship between Cd and sediment concentration could not be run because all of the Cd sediment values were below the detection limit.

Metal concentrations in sea cucumbers were used to determine human exposure from consumption (**Table 4.3**). To calculate the average amount of metal consumed as sea cucumber per kilogram human body weight per day, the average total weight of each tissue was calculated for all sea cucumber specimens collected from Tonga ($n = 55$) to give an average weight for each body part (body wall, respiratory tree, intestine, and gonad). From there, the overall average weights for respiratory tree, intestine, and gonads were summed, resulting in an average daily consumption weight of 15.9 g which was used as the weight of sea cucumber tissue consumed per day for subsistence purposes. This was based on the conservative assumption that those who rely on sea cucumber organs as a primary source of protein are consuming one average sized set of sea cucumber organs per day. The body weights used in these calculations were obtained from WHO (2011) for the average adult Tongan male body weight (95.7 kg) and the average adult Tongan female body weight (95 kg). For the commercial export fishery, the assumption was made that one sea cucumber per week was consumed, given the information which suggests that whole sea cucumbers are consumed as a singular dish in high-end markets, and restaurants in China (USDA, 2012), and based on a conservative estimate for weekly consumption. The weight of sea cucumber consumed per day was the average body weight measured of the Tongan sea cucumbers collected in the present study divided by seven (the number of days in the week) which was 6.3 g. The human weights used for the study on daily consumption of individual trace metals in Chinese consumers of Beche-de-mer (body wall) were 66.5 kg (men) and 56.8 kg (women) (Zhu, 1998). In the current study the total As (TAs) was measured, but As can be present in both inorganic and organic forms, and it is only the inorganic As which is considered toxic (Brooke & Evans, 1981). Consequently, a conversion factor (0.1%) was used to convert the total As into inorganic As (Schoof et al., 1999).

Table 4.3. Measured values of trace metals (mg kg⁻¹ wet weight) in sea cucumber tissues used in the human consumption risk assessment calculations.

Metal	Max. Organ	Average Organ	Max. Body Wall	Average Body Wall
Fe	76	21.1	43.7	10.5
Co	0	0.0	0.0	0.0
Ni	1	0.1	0.2	0.0
Cu	1	0.3	0.1	0.1
Zn	29	5.0	7.0	2.1
As	12	1.5	6.9	1.7

Max. organ refers to the maximum trace metal value found in either one of the three internal organs (respiratory tree, intestine, or the gonads); average organ refers to the overall average trace metal value of all three tissues for each respective metal; max. body wall refers to the maximum trace metal value found in the body wall; average body wall refers to the average body wall trace metal concentration for each respective metal.

4. 2. 4. Sediment Quality Guideline Quotients (SQGQs)

To quantify sediment pollution in a simple and concise way for each site, the sediment trace metal concentrations in the nearshore were used to calculate mean sediment quality guideline quotients (mSQGQ). Only nearshore samples were used to account for the fact that for the Atata site only nearshore samples were available, compromising other comparisons. The sediment trace metal concentrations were divided by their specified sediment quality guideline values (SQGs). The SQGs used for all metals except Ni were sourced from the Sediment Evaluation Framework (SEF) for the Pacific Northwest (2009), and for Ni the high ANZECC value was used (**Table 4.6**). The values were summed and then divided by the total number of trace metals to devise the mSQGQ (Long et al., 2006).

4. 3. Results

4. 3. 1. Sediment metal concentration

In 2014, the general order of sediment metals from highest to lowest in concentration were: Fe > As > Zn > Ni > Pb > Cu > Hg > Co. The only metal below detection limits was Cd. In 2015, the general order of sediment metals from highest to lowest concentration were: Fe > As > Zn > Pb > Ni > Cu > Hg. The metals which were below detection limits were Cd and Co. Overall, Tukatonga had consistently higher sediment trace metal concentrations relative to other sites.

Table 4.4. Sediment trace metal concentrations ($\mu\text{g g}^{-1}$) for the sampling season of 2014, categorised by distance from shore (DFS) for each sample.

Site	DFS	Fe	Co	Ni	Cu	Zn	As	Hg	Pb
Atata	Near	1389	0.3	1.4	0.7	1.5	5.8	0.3	1.0
Kolonga	Far	774	0.1	0.7	0.2	1.2	3.0	0.4	0.3
	Near	2313	0.4	1.1	1.9	6.6	4.6	0.3	2.2
Sopu	Far	1290	0.2	1.0	0.3	2.0	6.2	0.3	0.6
	Near	1448	0.2	1.0	0.6	2.0	5.9	0.3	0.6
Tukutonga	Far	2138	0.3	1.0	0.7	3.6	10.3	0.3	0.5
	Near	2651	0.4	1.2	1.1	7.7	10.8	0.4	1.2

Nearshore refers to the near-mid seagrass bed, and the farshore refers to the rubble/sargassum habitat.

Table 4.5. Sediment trace metal concentrations ($\mu\text{g g}^{-1}$) for the sampling season of 2015 categorised by distance from shore (DFS).

Site	DFS	Fe	Ni	Cu	Zn	As	Hg	Pb
Kolonga	Far	1007	0.9	0.3	1.8	4.5	0.1	0.4
	Near	2074	1.1	1.5	5.1	4.4	0.2	1.5
Sopu	Far	1406	1.2	0.5	1.6	6.1	BDL	0.5
	Near	1480	1.2	0.8	1.7	5.7	0.1	0.7
Tukutonga	Near	3499	1.5	2.3	47.0	14.1	0.2	2.2

No data is presented for Atata because it was not sampled in this season. Nearshore refers to the near-mid seagrass bed, and the farshore refers to the rubble/sargassum habitat. BDL = Below detection limits.

4. 3. 2. Sediment metal variation between years and sites

There was no significant difference in sediment Fe, Ni, Cu, Zn, As, and Pb concentrations between years ($p > 0.05$), however Hg concentrations were significantly higher in 2014 than 2015 ($p < 0.05$). The pollution indices (mSQGQ) for Atata, Tukutonga, Kolonga and Sopu were: 0.14, 0.14, 0.10, and 0.10, respectively, with $\text{Tukutonga} \approx \text{Atata} > \text{Kolonga} \approx \text{Sopu}$. Most trace metals were highest at Tukutonga, with Zn, Fe, and As sediment concentrations greater at Tukutonga than Atata, Kolonga, and Sopu (**Figure 4.1**). Additionally, Tukutonga

also had higher concentrations of Cu than Atata and Sopus (**Figure 4.1**). Kolonga had significantly higher concentrations of Cu than Atata (**Figure 4.1**). The concentrations of Ni, Hg and Pb did not differ significantly between sites.

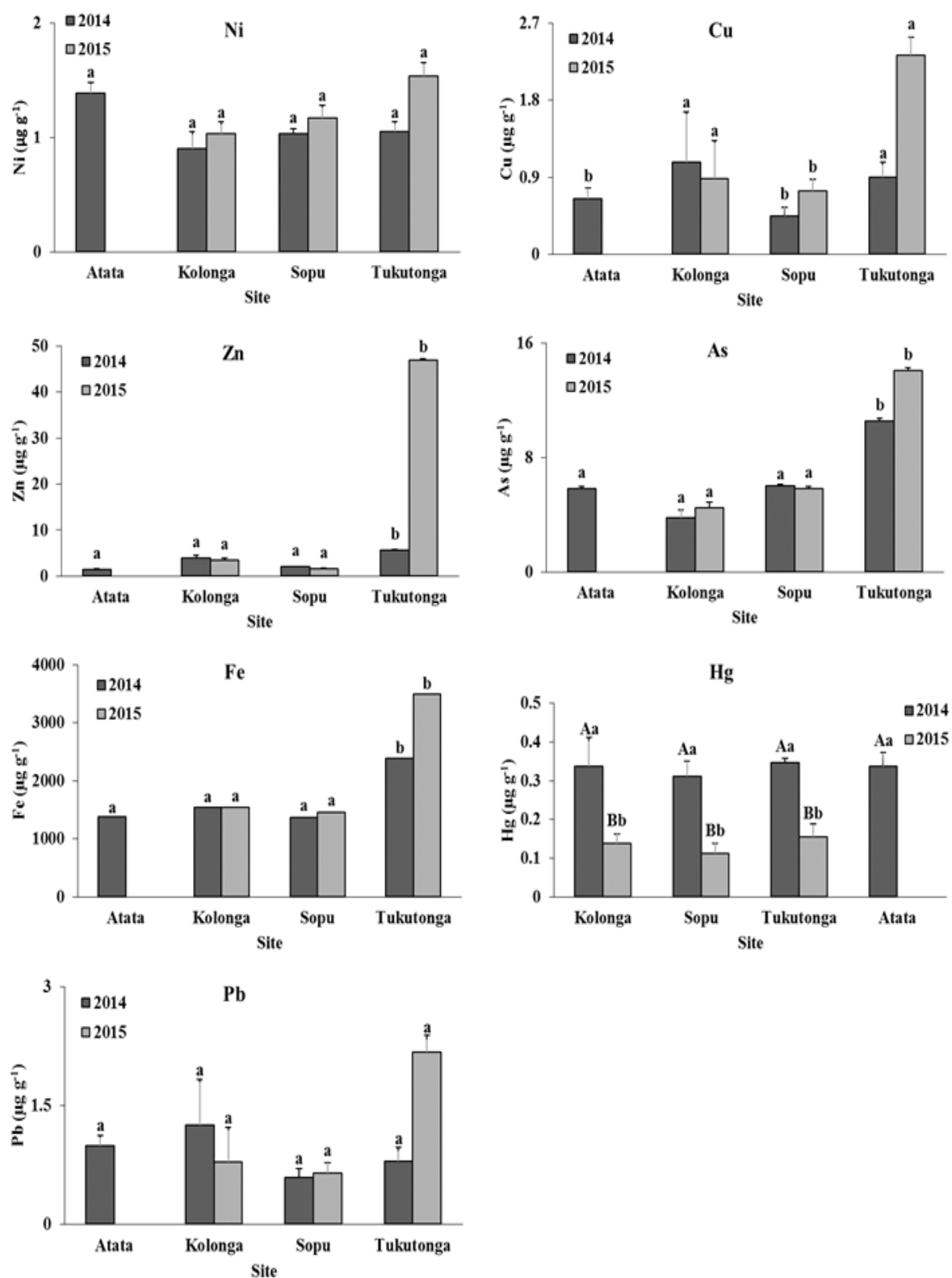


Figure 4.1. Sediment metal concentrations ($\mu\text{g g}^{-1}$) for the nearshore sediment samples from each site. Data presented here are raw, but were \log_{10} -transformed for analysis. Bars sharing lowercase letters were not significantly different in trace metals between sites within a year, and bars sharing uppercase letters were not significantly different in trace metals between years within a site.

4. 3. 3. Sediment metal variation as a function of distance from shore

The concentrations of Cu, Fe, Ni, Pb, and Zn were significantly higher in the nearshore than the farshore (**Figure 4.2**). The concentrations of As and Hg did not differ significantly between the nearshore and farshore environments (**Figure 4.2**).

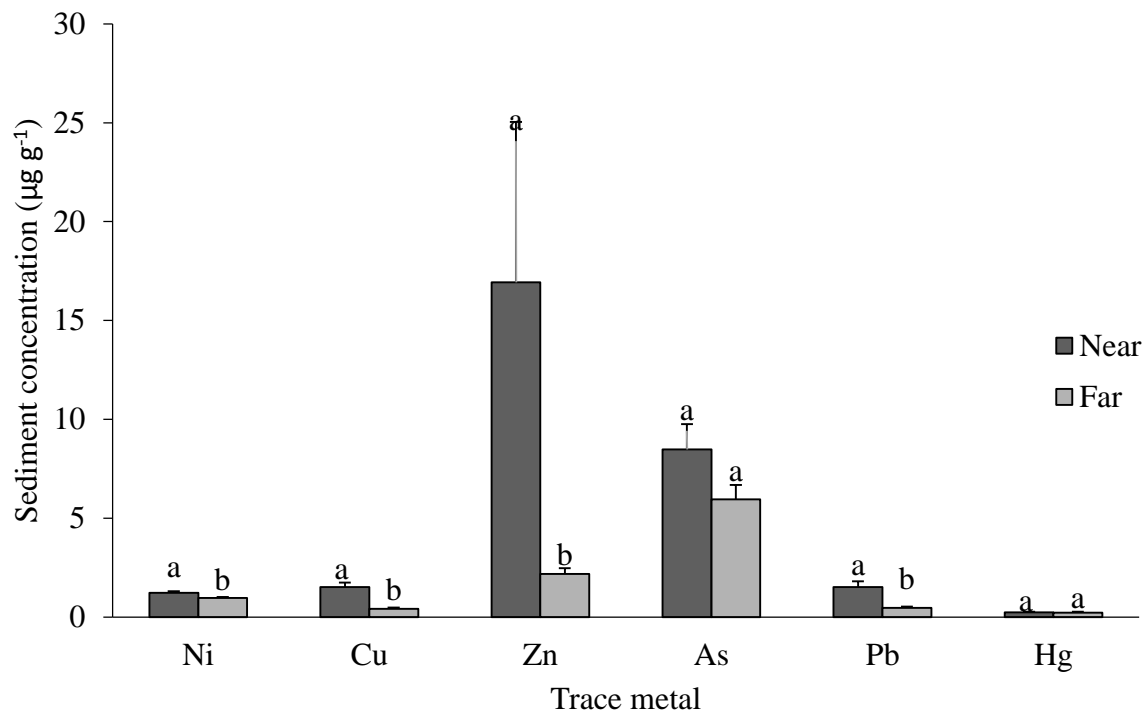


Figure 4.2. Trace metal concentration for each element grouped by distance from shore. Near ($n = 14$) and Far ($n = 13$). Bars sharing letters within an element are not statistically significantly different from one another as determined by a one-way ANOVA. Data displayed here are raw, but were \log_{10} -transformed for analysis.

4. 3. 4. Comparison of sediment metal concentrations to guidelines

The As, Cu, Ni, Pb, and Zn concentrations in the Tongan sediments were well below the low trigger values obtained from the EPA Sediment Evaluation Framework (SEF) for the Pacific Northwest (2009) and the Australia and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC, 2000) (**Table 4.4, Table 4.5, & Table 4.6**). The average As concentrations at Tuketonga were approaching the low trigger values (ANZECC, 2000) (**Table 4.4, Table 4.5, & Table 4.6**) While all Hg values were below the EPA guidelines and the high ANZECC trigger values, the majority exceeded the low ANZECC trigger values (ANZECC, 2000) (**Table 4.4, Table 4.5, & Table 4.6**).

Table 4.6. Guideline values for selected trace metals in sediments (mg kg⁻¹ dry weight).

Element	Safe concentrations (EPA)	Low (ANZECC)	High (ANZECC)	ISQG/TEL (Canada)
As	57	20	70	7.24
Cd	5.1	1.5	10	0.7
Cr	260	80	370	52.3
Cu	390	65	270	18.7
Pb	450	50	220	30.2
Hg	0.41	0.15	1	0.13
Zn	410	200	410	124
Ni	---	21	52	---

Sources: Sediment Evaluation Framework (SEF) for the Pacific Northwest (2009), the Australia and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC, 2000), and the interim marine sediment quality guidelines (ISQG)/threshold effect levels (TELs) from the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life (Canadian Council of Ministers of the Environment, 2001).

4.3.5. Tissue metal concentrations

The tissues analysed had metal concentrations that ranged between 10.01 – 551.30 µg g⁻¹ for Fe, below detection limits (BDL) – 0.65 µg g⁻¹ for Co, BDL – 10.69 µg g⁻¹ for Ni, 0.18 – 9.05 µg g⁻¹ for Cu, 3.80 – 315.90 µg g⁻¹ for Zn, 1.36 – 125.96 µg g⁻¹ for As. The majority of Cd values were BDL, and all tissue Hg and Pb values were BDL. There was no significant correlation between sediment and tissue metal concentrations for any of the metals and tissues studied ($p > 0.05$, $R^2 < 0.5$) (data not shown).

The maximum and average daily consumptions of Zn in sea cucumber internal organs (based on the assumption that one entire set of internal organs – respiratory tree, gonad, and intestines were consumed per day) and in the body wall (based on the assumption that one average sized sea cucumber body wall per week is consumed) exceeded the oral Reference Dose (RfD) for chronic Zn exposure (**Table 4.7**). The maximum daily consumption of inorganic As (IAs) and Ni in sea cucumber organs also exceeded the RfD (**Table 4.7**).

Table 4.7. Maximum and average amounts of each metal consumed per kilogram human body weight per day (wet weight) ($\text{mg kg}^{-1} \text{ day}^{-1}$) for males and females consuming sea cucumber organs (respiratory tree, gonad, and/or intestine) in Tonga (Traditional/Subsistence) and for those consuming the body wall in China (Commercial/Export), based on data collected from the four sites sampled in Tonga.

Metal	Traditional/Subsistence				Commercial/Export				RfD ($\text{mg kg}^{-1} \text{ day}^{-1}$)
	Max		Average		Max		Average		
	Male	Female	Male	Female	Male	Female	Male	Female	
Fe	13	13	4	4	4.11	4.81	0.98	1.15	---
Co	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	---
Ni	0.17	0.17	0.01	0.01	0.02	0.02	<0.01	<0.01	0.02 ¹
Cu	0.1	0.1	0.04	0.04	<0.01	<0.01	<0.01	<0.01	---
Zn	4.9	4.9	0.8	0.8	0.7	0.8	0.2	0.2	0.16 (men), 0.13(women) ²
TAs	2.0	2.0	0.3	0.3	0.7	0.8	0.2	0.2	
IAs	0.0019	0.0020	0.0002	0.0002	0.0007	0.0008	0.0002	0.0002	0.0003 ³

Oral Reference Dose (RfD) for chronic exposure according to the (United States (US) Environmental Protection Agency (EPA) guidelines for the daily intake of selected metals in milligrams per kilogram body weight per day ($\text{mg kg}^{-1} \text{ day}^{-1}$). References; ¹US EPA (1991); ² US EPA (2005); ³ US EPA (1995). TAs = Total As; IAs = Inorganic As, for measured values this was calculated using a conversion factor of 0.1% (Schoof et al., 1999). The maximum values presented here were calculated from the tissue (either respiratory tree, gonad or intestine) or the body wall with the greatest concentration of each particular trace metal measured in the current study. The average values presented here were calculated from the average tissue (respiratory tree, gonad, and intestine) or body wall trace metal concentrations measured.

4. 3. 6. *Stichopus horrens* – trace metal analysis

The following section is based on the analysis of only one species, *Stichopus horrens* (See Section 4. 2. 3.). The statistical analysis was performed on each particular tissue type separately to determine differences between sites without potentially confounding for differences in trace metal accumulation patterns between tissues. There were no significant differences in Fe, Co, Ni, Cu, Zn and As concentration between sites within tissues.

Cadmium concentrations were below detection limits (BDL) for all of the tissues analysed for *S. horrens*. The concentrations of trace metals were tissue-dependent for Fe, Co, Ni, Cu, Zn, and As.

Out of the four tissues analysed for trace metals, the tissue with the highest Fe concentration was the respiratory tree (**Figure 4.3**). Cobalt concentrations were highest in the respiratory tree and the intestine, and the concentrations of Ni and Zn were both significantly higher in the muscle bands than in the body wall (**Figure 4.3**). Similarly, Cu concentrations were

lowest in the body wall by comparison with the three other tissues (**Figure 4.3**). The highest concentrations of As were measured in the body wall and the respiratory tree (**Figure 4.3**).

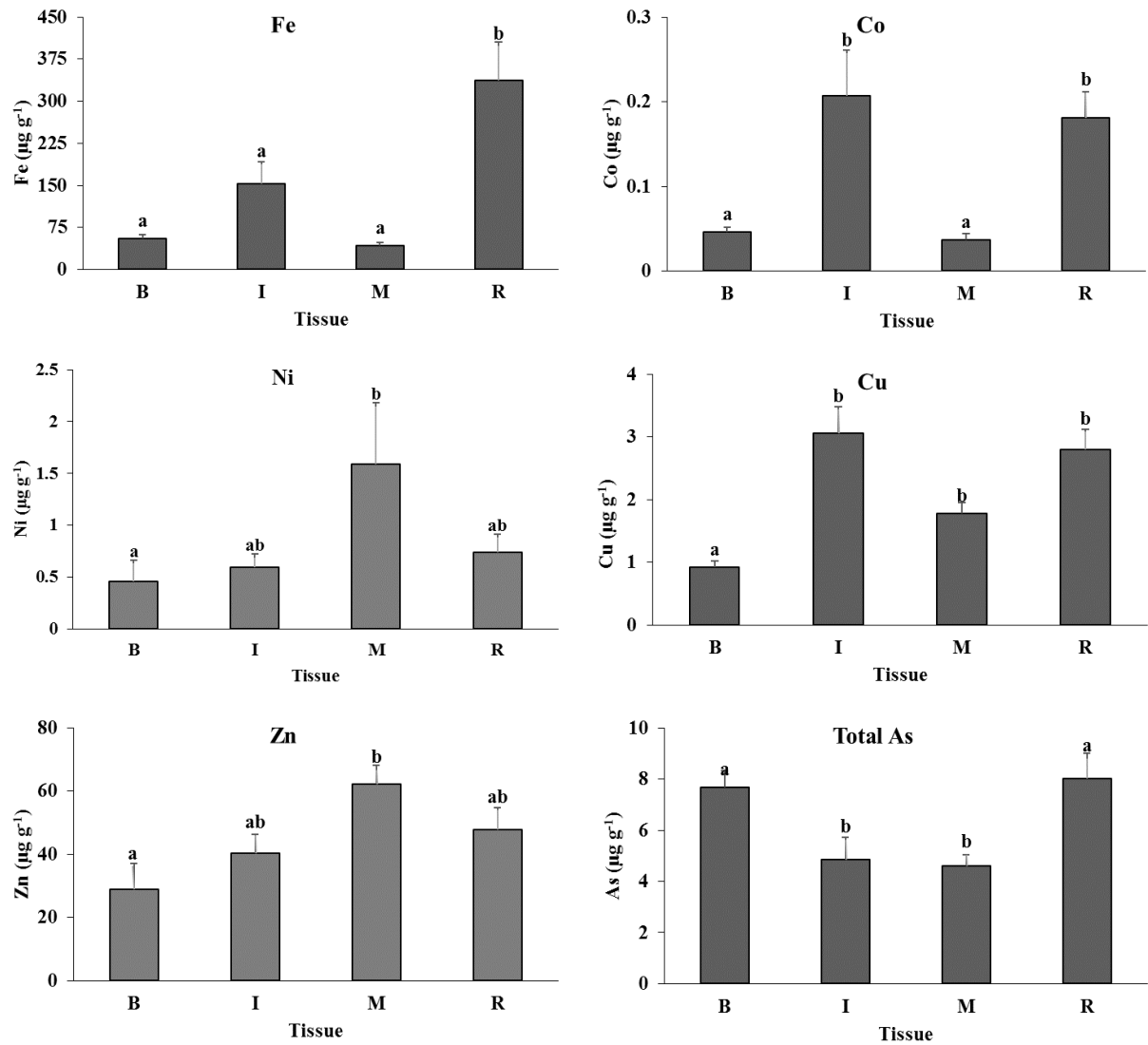


Figure 4.3. Average metal concentration ($\mu\text{g g}^{-1}$ dry weight) + standard errors, by tissue type (B is body wall, I is intestine, M is muscle band, and R is respiratory tree). Data displayed here are raw but were \log_{10} -transformed for analysis. Bars sharing letters are not statistically significantly different from each other as determined by a two-factor ANOVA and post-hoc Tukey test.

4. 4. Discussion

4. 4. 1. Sediments and tissues: a comparison with the literature

A large percentage of trace metals are associated with sediments, and therefore sediments represent a significant source of these contaminants in the marine environment (Temara et al., 1998). In the present study, the majority of sediment metal concentrations were below guideline concentrations. The highest concentrations of trace metals were generally found in the nearshore environment, with the exception of As and Hg which did not differ significantly between nearshore and farshore sediments. The concentrations of some metals detected in sediments were similar to those reported in the literature for the southwest Pacific. For instance, the concentrations of As, Pb, and Fe in the current study were similar to those previously described in Fanga'uta lagoon in Tonga by Morrison and Brown (2003). However, the concentrations of Ni, Cu, and Zn were much lower in the present study than those described for Fanga'uta lagoon (Morrison & Brown, 2003). The concentrations of sediment Zn and Cu in the present study were much lower than the concentrations described in the intertidal zone in Fiji, but the Hg concentrations were similar (Morrison et al., 2001). Additionally, another study conducted in Fiji (in the Great Astrolabe Lagoon) demonstrated lower marine sediment concentrations of Co, Cu, Zn, Ni, and Pb than those which were measured in Tongan sediments (Morrison et al., 1997). Furthermore, the concentrations of Zn and Co were lower in Tongan sediments compared with mangrove sediments in New Caledonia, but the concentrations of Fe, Cu, and Ni were compatible between the two studies (Marchand et al., 2011). The concentrations of trace metals in sediments measured in the present study are consistent with those reported in the literature, although some differences do exist as discussed above.

These differences are likely to reflect variation between organic content of the sediments, differences in grain sizes, and/or differences in contamination sources between the studies discussed above. Dissolved organic matter can bind to trace metals, making them less bioavailable within a system (Reuter & Perdue, 1977). Additionally, grain size can influence the trace metal concentrations of sediments, with finer grains generally acting as transporters of trace metals (Rubio et al., 2000; Ip et al., 2007). The distance from contamination source can also influence trace metal concentrations, with trace metals decreasing in concentration with increasing distance from site (Flammang et al., 1997; Greaney, 2005; Denton et al., 2009). Thus, it is possible that the differences in sediment metal concentrations demonstrated

by the present study are reflecting variation in site-specific factors such as sediment grain size, organic matter content, and the proximity to contamination sites.

The concentrations of trace metals measured in the sea cucumbers in the present study are comparable to trace metal concentrations previously reported for tissues of *Holothuria edulis*, *Holothuria atra*, *Bohadschia marmorata*, *Stichopus chloronotus*, *Holothuria scabra*, and *Bohadschia* sp. (Denton & Morrison. 2009; Denton et al., 2009; Jinadasa et al., 2014). Trace metal concentrations of Tongan sea cucumbers were also within a similar range to the species sampled for metals from a supermarket located in Guangzhou, China (Wen & Hu, 2010).

In comparison with other benthic-dwelling species found in the same region (Southwest Pacific) as Tonga, some similarities and differences in trace metal accumulation are demonstrated. For example, the concentrations of tissue Fe measured in the present study were similar to those of the mollusc *Nautilus macromphalus* in New Caledonia, (Bustamante et al., 2000). However, the concentrations of Ni, Cu, Co, and Zn measured in the current study were lower than those described by Bustamante et al. (2000) for *N. macromphalus*. While the tissue concentrations of Cu measured in the present study were comparable with those measured for the tumid venus clam *Gafrarium tumidum* in Fanga'uta lagoon, Tongatapu, the concentrations of Cd, Hg, and Pb reported in the clam were relatively high by comparison with the sea cucumber tissues in the present study (these tissue metals were deemed below detection limits in the current study) (Naidu et al., 1991). In addition, the concentrations of Zn, Cu, and As measured in sea cucumber tissues were similar to those measured in the cockle *Anadara antiquate* and *Anadara* sp. in Fiji, but not for the other metals studied (Morrison et al., 1997; Morrison et al., 2001). The concentrations of Zn reported in sea cucumber tissues in the current study are similar to other Zn values reported in the literature for the mussel *Mytilus galloprovincialis*, the echinoid *Diadema setosum*, and the sea star *Asterias rubens* (Flammang et al., 1997; Beiras et al., 2003; Danis et al., 2004).

It is possible that the differences in trace metal concentrations between sea cucumbers measured in the present study, and other benthic-dwelling marine species sampled in the Pacific by other studies, may be due to inter-specific variation in feeding strategies, body size and life span, and differing nutrient requirements between species. Inter-specific differences in trace metal accumulation can result from different feeding mechanisms between species (Lee et al., 1998). A study by Reinfelder et al., (1997) demonstrated that retention times of trace metals in the deposit-feeder *Macoma balthica* were greater than those

for the other suspension-feeding species in the study. Thus, inter-specific differences in trace metal accumulation are to be expected, particularly given the different feeding mechanisms of the species compared in the preceding paragraph.

Environmental conditions such as temperature and salinity can also influence trace metal uptake (Jackim et al., 1977; Park et al., 2014). In addition, body size, life span and growth rate can affect the bioaccumulation of trace metals (Phillips & Rainbow, 1993; Dobicki & Polechonski, 2003; Vieira et al., 2011). Different animals also have different nutrient requirements, which could influence the inter-specific concentrations of nutrient metals such as Cu and Zn through specific dietary pathways. This has been demonstrated in Eiders (seabirds) for which elevated basal Cu concentrations were attributed to the consumption of a food source which had the Cu-containing blood pigment haemocyanin (Savinov et al., 2003). Thus, in organisms which have different respiratory pigments, such as haemoglobin (Fe-containing) or haemocyanin (Cu-containing) (Manwell, 1960) a difference in the natural concentrations of occurrence of each of these metals would be expected. For example, sea cucumbers generally employ haemoglobin as a respiratory pigment, while most mollusc and crustaceans use haemocyanin (Withers, 1992). Thus, it is possible that local conditions such as temperature and salinity, diet and blood pigment type, and differences in body size and life history strategy could be contributing to the differences in bioaccumulation between species discussed above.

4. 4. 2. Sediments

The decrease in trace metals in the farshore compared to the nearshore demonstrated in the present study is in agreement with the findings of other studies. A decrease in sediment trace metal concentration with an increase in distance from the site of contamination has been demonstrated with distance from cities, distance from rubbish dumps and distance from shore (Flammang et al., 1997; Greaney, 2005; Denton et al., 2009). Thus, the higher concentration of metals in sediments in the nearshore compared with the farshore demonstrated in this study, is likely due to anthropogenic trace metal inputs. However, it is also possible that this pattern arises from natural metal inputs from mineral-rich soils such as mangrove sediments (Harbison, 1986). The lack of a difference in As and Hg between nearshore and farshore sediments suggests that these two metals are naturally present in marine sediments around Tonga, possibly due to volcanic activity in the region (Signorelli, 1997; Ferrara et al., 2000; Wei & Wang, 2011). The sediment concentrations of Hg exceeded the low trigger values but

below the high trigger values (ANZECC, 2000), and it is possible that volcanic activity in the region is contributing to this.

In the present study, Tuketonga had consistently higher sediment trace metal concentrations than nearly all of the other sites in the nearshore environment. It is possible that trace metal accumulation at Tuketonga is due in part to its proximity to Fanga'uta lagoon. Within Fanga'uta lagoon catchment, substantial development and urbanisation has resulted in the discharge and leakage of sewage and hospital waste into the lagoon (Naidu et al., 1991). Elevated nutrient concentrations in the lagoon have been demonstrated, and are likely to be a product of sewage leakage from septic tanks and pit latrines (Naidu et al., 1991; Newton, 2008). Historically, Tuketonga has been the site of a quarry, a place where solid waste has been deposited in mangroves, and where sludge-drying beds and a sewage plant have been present (Chesher, 1984). It is unclear whether these sources of pollution are still present in the area.

4. 4. 3. Correlation between sediment and tissue metals

A lack of correlation between sea cucumber tissue metal concentration and sediment metal concentration demonstrated in the present study and other research indicates that sea cucumbers do not reflect sediment metal concentration (Xing & Chia, 1997; Denton et al., 2009). This suggests that the diet (sediment ingestion) may not be a significant exposure route of sea cucumbers to trace metals. However, it has also been suggested that sea cucumbers are able to regulate tissue metal concentrations, with evidence existing for Zn and Ni (Denton & Morrison, 2009). The capacity for organisms to regulate trace metal uptake is variable. For example, the crab *Carcinus maenas* is able to regulate its Zn and Cu uptake until a certain threshold level, while the barnacle *Elminius modestus* is unable to regulate uptake of these metals (Rainbow, 1985). Thus it is possible that the lack of correlation between tissue and metal trace metals seen in the present study is due to the internal regulation of tissue metal concentrations. However, while it is likely that sea cucumbers are capable of regulating trace metal uptake, it is unlikely that they are able to do so for all metals. It is possible that sea cucumbers are able to regulate tissue trace metal concentrations for the relatively non-toxic essential metals for which the correlation analysis was performed on, but not for the more toxic metals such as Pb, Hg, and Cd. This lack of regulation has been demonstrated in the crab *C. maenas* which was able to regulate Cu and Zn uptake but not Cd (at any concentration); in prawn larvae *Palaemon serratus* which were able to regulate Zn but

not Cd uptake; and in the freshwater fish *Tilapia zilli* which did not accumulate Zn but did accumulate Cu, Cd, and Pb following exposure (Devineau & Triquet, 1985; Rainbow, 1985; Kalay & Canli, 2000). However, it is possible that dissolved metals represent just as an important exposure pathway as dietary metals.

Dissolved trace element concentrations were not measured in the present study, but marine organisms are capable of absorbing metals in this form. The sea cucumbers sampled in the present study all occupied benthic sediments, where they can be exposed to the elevated concentrations of trace metals associated with interstitial water (Brumsack & Gieskes, 1983). The uptake of dissolved trace metals has been demonstrated in the sea urchin *Paracentrotus lividus*, the polychaete *Nereis succinea*, and crustaceans (Rainbow, 1997; Wang et al., 1999; Radenac et al., 2001). Additionally, dietary metals may be less bioavailable than dissolved metals because they can complex with organic matter (Mason, 2013). Organic matter is often deposited in marine sediments (Burdige, 2006), and thus it is likely that trace metals in the diet (sediment) of sea cucumbers are less bioavailable due to complexation with organics. However, digestive processes can alter speciation and increase bioavailability through solubilisation in the gut (Chen & Mayer, 1999). Thus, it is possible that dietary components other than sediments, are influencing the lack of correlation between sediment and sea cucumber metal concentrations. For example, macroalgae is known to have a relatively high As content (Laparra et al., 2003). It is possible that because of the low algae:sediment ratio sampled in the present study, the relationship between sediment/algae concentration and sea cucumber tissues was not identified. In summary, it is possible that internal regulation by sea cucumbers of metal uptake, a reduced bioavailability of trace metals in sediments due to complexation, and/or the importance of the dietary vs. dissolved trace metal exposure route may be influencing the lack of a detectable relationship between sediment and tissue trace metal concentrations seen in the present study.

4. 4. 4. Differences between tissue types

The differences in metal accumulation between tissues observed for *Stichopus horrens* in the present study are aligned with other studies in the literature which have also demonstrated tissue specific trace metal accumulation. For example, the starfish *Asterias rubens* exhibits differences in Cd, Pb, and Zn accumulation between body compartments (Temara et al., 1998), and in the echinoid *Diadema setosum* Fe, Zn, and Cu concentrations were higher in the gonads than in the spines and body wall (Flammang et al., 1997). Additionally, the

intestinal tissue of *Paracentrotus lividus* had the highest accumulation of Cd, while the gonads, body wall, Aristotle's lantern and the coelomic fluid had decreasing concentrations of Cd by comparison (Warnau et al., 1997). In sea cucumbers, tissue specific trace metal accumulation has also been demonstrated. For example, Cr partitioning between tissue types has been demonstrated in the sea cucumber species *Bohadschia marmorata*, *Bohadschia argus*, and *Holothuria atra* (Denton et al., 2009). The accumulation of Fe in *S. horrens* was highest in the respiratory tree which most likely reflects Fe bound to haemoglobin in this tissue (Herreid et al., 1976; Jangoux & Lawrence, 1982; Baker, 1988; Jaeckle & Strathmann, 2013).

The patterns of tissue specific trace metal accumulation demonstrated by *S. horrens* may indicate tissues which could present the greatest consumption risk if concentrations were elevated. These tissues are: the muscle bands for Ni; intestines, muscle bands and respiratory tree for Cu; respiratory tree for Fe; intestines and respiratory tree for Co; intestines, muscle bands and respiratory tree for Zn; body wall and respiratory tree for As. It has been suggested that different tissues can have different retention times for metals and can thus indicate long vs short-term metal accumulation (Temara et al., 1998). Therefore, further investigations into which tissues are indicative of short- vs. long-term metal accumulation in sea cucumbers could provide useful information in terms of their capacity for use as a bioindicator.

4. 4. 5. Trace metal - human health risk assessment

For the following section, As, Zn, and Ni are the only metals discussed in detail in terms of their potential health risks to human consumers of sea cucumber tissues. This is because they were the only metals that exceeded the RfD values based on the assumptions of this study. In the current study, the maximum daily intake concentrations of Ni and inorganic As for Tongan adults were above the RfD values (**Table 4.7**). Additionally, the maximum daily intake concentrations of inorganic As for Chinese consumers of body wall exceeded the RfD values, while the maximum concentrations of Ni were equal to those RfD guidelines (**Table 4.7**). Thus, the consumption of sea cucumber organs and/or the body wall with the maximal concentrations of Ni and inorganic As concentrations measured in the current study may pose a human health risk. The groups most at risk are those which consume sea cucumber tissues on a frequent basis and in greater quantities than taken into account by the current study (> 1 respiratory tree, intestine and gonad per day in Tongan populations, or > 1 serving of sea cucumber body wall per week in Chinese populations). When interpreting these results, it is

important to consider other routes of As and Ni exposure such as: the air, inhalation of contaminated dust, the ingestion of contaminated drinking water, and the amounts of other foods eaten which may be high in As or Ni (Farmer & Johnson, 1990; Cempel & Nickel, 2006; Sirot et al., 2009; Smith & Steinmaus, 2011). Thus, a greater understanding of the other Ni and As exposure routes in Tongan and Chinese populations would be beneficial when quantifying the risk of human health effects due to chronic As exposure or Ni exposure from the consumption of sea cucumbers harvested from the sites sampled in Tonga. Furthermore, surveys in Tonga quantifying the actual quantity of sea cucumber consumed on a daily or weekly basis by those who rely on this as a significant protein source would provide useful information when determining the risk factor for subsistence fishers in the Tongan region. These results should be interpreted with caution because of the assumptions about weight and daily sea cucumber consumption quantities made in this study.

In the present study, both the maximum and average concentrations of Zn consumed on a daily basis for traditional/subsistence and commercial/export purposes were above the daily consumption guidelines (**Table 4.7**). Although Zn is considered relatively non-toxic (National Research Council (US) Safe Drinking Water Committee, 1980), it can have human health effects when consumed at excess concentrations. The most at risk group of people from Zn overexposure are those consuming the internal organs and body wall at quantities within and above those in the current study. As with As, it is important to consider other factors considering the human health risk that sea cucumber tissues may pose to Tongan communities, and the consumers of the export products, as well as other exposure sources of Zn such as atmospheric, occupational, water, and dietary sources.

Future recommendations

Given that trace metals are sourced from both natural and anthropogenic inputs (Sañudo et al., 1991), I would suggest that future studies investigate the primary source of trace metals around Tongatapu. Furthermore, the conversion factors for total to inorganic As used in the present study are based on findings for species other than sea cucumber, thus the results presented here may not be completely accurate. Future studies should investigate the relationship between inorganic and organic As in sea cucumber tissues.

Summary

The present study indicated that the concentrations of trace metals in coastal marine sediments around the island of Tongatapu fell within the guidelines for the protection of both

human and aquatic health. There did appear to be anthropogenic inputs of trace metals into the marine system indicated by the relatively higher concentrations of trace metals in the nearshore compared to the farshore sediments. There were inter-site differences in pollution concentrations, with Tukumotu having the highest trace metal concentrations, likely related to urbanisation. Additionally, there was no correlation between sediment and sea cucumber tissue metal concentrations.

According to the findings of this study, there were some groups of people who may be at risk of excess intake of Ni, As and Zn if they consume sea cucumbers above the daily quantities used in this study. The people most at risk are those who are heavily reliant on sea cucumber as a primary protein source in Tonga (traditional/subsistence) as well as those consuming Beche-de-mer in the quantities of greater than 1 sea cucumber per week in China.

5. General Discussion

5.1. Regeneration

The primary goal of the regeneration study (Chapter 2) was to determine if the use of traditional fishing techniques has an effect on resource allocation in sea cucumbers. This study demonstrated that the respiratory tree and intestines were the first organs to regenerate, and that regeneration of gonads may be delayed following excision and/or evisceration.

Gamete resorption can be a mechanism by which to obtain energy for metabolic processes (Herlin-Houtteville & Lubet, 1975; Pipe, 1987; Shumway & Parsons, 2011). This information combined with the literature detailing that gonads are the last organ to regenerate following evisceration or excision (Kille, 1935; Kille, 1942; Drumm & Loneragan, 2005) suggest that the regeneration and/or development of gonads is energetically costly. The formation of gametes (gametogenesis) requires a significant nutrient supply either from existing tissues or from a food source (Pipe, 1987). The regeneration study demonstrated that different substrates (protein vs. carbohydrates) were used to fuel different life processes; with protein being the primary fuel for regeneration and a mixture of protein and carbohydrates fuelling organ maintenance. Thus, it seems that nutrient catabolism, and the types of nutrients required for different processes, may change depending on the energy requirements of the animal. Thus, the energy which would otherwise be used to fuel gonad development may be instead directed toward the regeneration of other internal organs. This has been discussed by Drumm and Loneragan (2005) who suggested that gonad removal during periods of reproductive activity may cause delayed spawning.

Differences in organ regeneration time between animals has been attributed to a number of factors, with the number of times that an animal has lost its internal organs or been previously wounded, among them (Needham, 1952). The effects of repeated regeneration on organisms has an effect on their fitness. For example, decreased growth has been observed in

polychaetes that have been repeatedly injured (Lindsay, 2010). Furthermore, the frequency of injury in coral colonies is closely related to colony decline, although the ability of corals to asexually reproduce can act to mitigate the effects of environmental disturbance (Hughes & Jackson, 1985; Cumming, 2002). Thus, it would be useful to conduct studies to determine the frequency of regeneration which sea cucumber species can survive, and the role of asexual reproduction in mitigating the effects of injury. Rotational fishing schemes for the traditional fishery in Samoa have been proposed by Eriksson (2006) as a means by which sea cucumber populations can recover from organ harvesting before they are cut again. A study into the effects of multiple organ harvesting, and the length of time required for organisms to recover from cutting would provide useful information when designing and implementing such a regime.

Over the course of the regeneration research, it asexual reproduction occurred in *S. horrens*. Furthermore, it became evident that traditional fishing techniques may act as a trigger for fission. The harvesting of internal organs is likely a stressful event, which may represent a 'disturbance' and could thus be categorised as such. Disturbance has been shown to trigger colony growth in the coral *Plexaura kuna* via asexual propagation (Coffroth & Lasker, 1998). Furthermore, fission rates have been attributed to anthropogenic and natural reef disturbances in populations of *Holothuria atra* in the Indo-Pacific (Conand, 1996). Thus, in areas which are more disturbed such as those experiencing high levels of traditional fishing pressure, elevated rates of fission are likely, and it is possible that this may come at a cost to genetic diversity.

There are many advantages to sexual reproduction which arise through genetic recombination. Genetic recombination is the process of the exchange and rearrangement of genetic material during fertilisation, which can reduce and eliminate deleterious mutations and result in elevated population genetic variability through the generation of new genotypes (Rice, 2002; Grapputo et al., 2005; Barbuti et al., 2012). Populations with high genetic variability have greater fitness than those with reduced genetic variability, and are better adapted to respond to environmental change (Crow, 1992). There are also benefits to asexual reproduction because populations are able to propagate new genotypes asexually, and this can also be a mechanism important in population maintenance (Barbuti et al., 2012; Thorne & Byrne, 2013). In addition to sea cucumbers, there are many organisms which are able to reproduce both asexually and sexually, such as the cyprinid fish *Carassius gibelio*, the aphid *Rhopalosiphum padi*, and the sea anemone *Actinia tenebrosa* (Ayre, 1984; Delmotte et al.,

2002; Barbuti et al., 2012). Given the seasonality of asexual reproduction demonstrated by *Holothuria atra* and the sea star *Nepanthia belcheri* (Ottesen & Lucas, 1982; Lee et al., 2008b) it is likely that sexual and asexual reproduction vary in their importance for population maintenance depending on environmental condition and time of year. It would be beneficial to understand the ‘normal’ baseline levels of asexual reproduction in a population disturbed primarily by environmental factors alone, and to compare these levels to populations experiencing ‘higher’ levels of disturbance by traditional fishing or anthropogenic means. This could be achieved by simple ecological surveys documenting asexual and sexual propagules, an example of which has been demonstrated in Thorne et al. (2013). In addition to this study, research of additional interest could include the use of genetic techniques such as the analysis of microsatellites, to determine differences in genetic variability between populations with high levels of asexual reproduction compared to those with lower levels (Delmotte et al., 2002). These studies could provide useful information on the mechanisms of population reproduction and the role of asexual reproduction in population maintenance and the response to disturbance.

5. 2. Methodological considerations of the regeneration study

Stocking density can effect stress levels in sea cucumbers (Pei et al., 2012). Pei et al. (2012) conducted experiments that showed a stocking density of 8 animals in a 40 litre aquarium could be maintained without causing excessive stress to the animals (i.e. elevating coelomic fluid cortisol and lactate levels and decreasing glucose levels). In the current experiment, the bags were 1.3 l in volume, resulting in a stocking density of 8 animals per 1.3 l, even though the bags were kept in a tank with a much larger water volume of water than this. The initial experimental set-up was unsuccessful due to specimens escaping through the wire mesh dividers in the tank. Thus, the ‘bag’ approach was taken because of the variable sea cucumber density measured in the field, with many observations of crowded aggregates, in combination with time and resource constraints. In addition, a control treatment was used in which the animals were measured for coelomic fluid glucose and lactate concentrations as indicators of stress in the experimental setup.

It is possible that the animals in the study were stressed due to relatively high stocking densities. However, any stress created by the experimental set-up was accounted for by the controls, and by comparing the sham and excised treatments to the controls. In addition, the parameters that were measured in this study to quantify the effects of the experimental

treatment (coelomic fluid lactate and glucose) can be indicative of a secondary stress response (López-Patiño et al., 2013). No lactate was found in either the body wall or coelomic fluid of the controls, or any of the animals in this study, which indicates that they were not anaerobically metabolising. Other studies have demonstrated that glucose levels increase during periods of anaerobic activity to produce greater amounts of lactate (Barnett & Pankhurst, 1998; Bergmann et al., 2001; Lund et al., 2009). The low levels of metabolised glucose, combined with low coelomic fluid and body wall lactate concentrations demonstrated in this study (See Section 2.3.4.) indicates that the animals were not anaerobically respiring. Anaerobic respiration can be indicative of oxidative stress, and is a mechanism by which organisms can reduce the associated cellular damage (Ju et al., 2014). I would suggest that future studies quantifying stocking density effects on these animals' measure cortisol levels, because cortisol levels can be used as a primary indicator of stress (Sumpter, 1997). Therefore, it cannot be entirely discounted that the animals may have been stressed, but stress (in the form of anaerobic respiration) is not demonstrated by the physiological end-points that were measured in this study. For future studies, however, I would suggest constructing much larger shade cloth bags reinforced with plastic piping to form cages and prevent the shade cloth collapsing. I would also investigate tagging techniques, although the outcomes of tagging experiments have been variable (Purcell et al., 2009; Davey et al., 2011).

5.3. Ecology

The ecology section of this research indicates that the area of Kolonga has suffered high intensity fishing pressure, and that the populations of sea cucumbers in this area have been unable to recover. In addition, the presence of a brown film of algae over the sediment and seagrass epiphytes, combined with the hydrogen sulfide odour of the sediment at this site suggests that nutrient loading is also occurring, which could also be influencing the recovery of sea cucumber populations in this area. Nutrient enrichment caused by anthropogenic inputs can result in the decrease of coral reefs (D'Angelo & Wiedenmann, 2014). It has been suggested that higher sea cucumber diversity could be related to the presence of an intact fringing reef (Yuval et al., 2014). Thus it is possible that nutrient enrichment may be influencing coral reef structure, and the abundance of sea cucumbers in turn. In addition to coral reef structure, the epiphytes resulting from nutrient loading can affect light attenuation in seagrass beds and thus can negatively affect their productivity (Short & Wyllie-Echeverria, 1996). As discussed in Chapter 3 (Ecology), sea cucumbers can have a positive effect on

seagrass productivity, thus the loss of these animals from this ecosystem may be contributing to the possible nutrient loading at Kolonga, thus instigating a cycle by which the lack of sea cucumbers contributes to elevated nutrients resulting in the degradation of the marine environment, and thus discouraging sea cucumber settlement due to unfavourable conditions.

As discussed above, the effects of nutrient loading, on nearshore environments can result in a loss in seagrass productivity and overall habitat degradation. Nutrient enrichment can result in the formation of toxic phytoplankton blooms, and the loss of coral reef species (Smith et al., 1999). Furthermore, eutrophication and the subsequent algal blooms which follow have been attributed to mortalities in shellfish (Shumway, 1990). In a terrestrial-aquatic example, benthic community stream diversity, abundance and biomass was reduced in areas polluted by nutrient run-off (Lemly, 1982). Thus, nutrient loading can have negative effects on community composition, survivorship, and can contribute to changes in habitat. Sea cucumbers can control nutrient loading by reducing the amount of dead organic matter in the benthic environment, and can thus decrease anaerobic processes in sediments and restrict eutrophication (Michio et al., 2003). Nutrient loading and overfishing can result in habitat degradation (Jackson et al., 2001; Lotze et al., 2006). Habitat degradation has contributed to oyster decline in Chesapeake Bay (USA), and is a factor threatening fish populations (Rothschild et al., 1994; Musick et al., 2000). Thus, it is important to determine the effects of elevated nutrients on benthic-dwelling communities in coastal environments, how this may contribute to the degradation of nearshore habitats, and the effects that this may have on sea cucumber survivorship and diversity. This would assist in determining whether elevated nutrient levels may be reducing sea cucumber densities in nutrient-enriched areas. It is important to understand this, because low densities of sea cucumbers can greatly influence their reproductive success.

Sea cucumbers are gonochoric broadcast spawners, meaning that individuals are either one sex or another and release their gametes into the water where fertilisation occurs. Thus, for sexual reproduction to occur, they need to be relatively close to each other (Purcell et al., 2009). The success of reproduction in sea cucumbers is thought to be elevated by high densities of individuals, short distance between animals and large organisms (Eriksson et al., 2010). In natural populations which have become too sparse to effectively reproduce, their ability to recover from overfishing is poor. It is likely that sea cucumber populations at Kolonga are too small to successfully reproduce and thus to recover without human intervention. In this instance, restocking of natural populations from hatchery reared juveniles

may be necessary (Mills et al., 2012). Restocking is most successful when localised high densities of spawning individuals are created, and act as a larval source for other populations (SPC & WorldFish, 2009; Mills et al., 2012). Thus, such aggregations should be placed in areas where currents may disperse larvae to other settlement areas (SPC & WorldFish, 2009; Mills et al., 2012). However, restocking alone is not necessarily guaranteed to be successful (SPC & WorldFish, 2009). To ensure successful restocking and the propagation of wild populations, other management measures need to be put in place simultaneously such as: no-take zones, size restrictions, penalties for those that do not comply with regulations, and regulation of the equipment that can be used for fishing activities to protect the released animals (Purcell & Kirby, 2006; SPC & WorldFish, 2009). The current moratorium in Tonga is in place for the next 5 years. I would suggest that ongoing monitoring be undertaken to assess how sea cucumber populations recover during this time, and if recovery rates are low, that restocking and further management measures such as those discussed above be put in place. In addition, I would also suggest that the nutrient inputs at Kolonga be reviewed and some changes be made in an attempt to restore the seagrass habitat in this area and reduce the nutrient loading evident in this ecosystem. This is likely to make any restocking efforts more successful, and in turn, sea cucumbers would be able to act as a buffer to nutrient loading in the future (Vandover et al., 1992; Slater & Carton, 2009; Hannah et al., 2013).

Prior to commencing a restocking project, it is important to assess how other management measures may effectively contribute to sea cucumber population propagation. Such measures include moratoria and/or the artificial formation of spawning aggregations of wild individuals and their subsequent protection (Bell & Nash, 2005). At the present time, commercial fishing activities are regulated under a moratorium in Tonga, and preceding this there were size restrictions, fishing seasons, and a ban on the use of underwater breathing apparatus for fishing purposes, stipulated by the Government of Tonga (1994). However, illegal sea cucumber fishing has been documented in Tonga and it is possible that this is also contributing to the depletion of stocks, as regulatory measures such as the ban on underwater breathing apparatuses in Tonga and the implementation of the Fishery Management Plan were less successful than originally hoped (Pakoa & Bertram, 2013; Pakoa et al., 2013). Thus, given that past regulatory measures in Tonga have failed to maintain sea cucumber populations in this region, it is possible that more drastic measures such as restocking in areas which are most depleted (such as Kolonga) are required.

The results of the ecological research, specifically the sizes of the sea cucumbers at the sites sampled in Tonga suggest that a review of the size limits for all species may be necessary. Furthermore, a comparison should be made with the size limits in other countries, and the size at reproduction for each species. The current size restrictions in Tonga for *Holothuria atra* are 16.5 cm (wet) which is aligned with the reported value of first sexual maturity for this species from New Caledonia, but smaller than that for Fiji (See Section 3.4.4.) (Government of Tonga, 2008). For *S. horrens* the size restrictions stipulate a minimum length of 12 cm, which is much smaller than the size at sexual maturity of 25 cm, and also smaller than the size restrictions for this species in Australia (See Section 3.4.4.). Thus, I believe that a review of the current size restrictions on the sea cucumber species around Tonga may be required, which may encourage future sexual reproduction in these animals by promoting their growth to sexual maturity before being removed from the ecosystem.

The recent moratorium and the past attempts at fisheries management in the forms of underwater breathing apparatus bans and size regulations are all steps in the right direction; however, implementation of these measures in a country where resources and funds are low is complex. Tonga is a low income country and 22.5% of Tongans were living below the National Basic Needs Poverty Line in 2009 (Fifita, 2011). Thus, the implementation of community-based programmes in such a situation is likely to be the most feasible mechanism by which to regulate sustainable sea cucumber harvesting practices. The success of community-regulated areas has been demonstrated in Fiji, where the locally managed marine area approach has resulted in greater fish catches, an increase in clam harvesting and an overall elevation in household income (Meo, 2012). It is also likely that the Special Management Area (SMA) at Atata in the Kingdom of Tonga is also exhibiting the positive effects of locally-based management measures (See Section 3.4.1.). Community-based management has also been shown to reduce theft of sea cucumbers from sea-ranches in Madagascar, through local farmers taking it upon themselves to rotationally guard the resource (Rougier et al., 2013). Furthermore, the use of sea-pens in sea cucumber production allows the involvement of the community at an early stage (the rearing of juveniles in the sea-pens) (Mills et al., 2012). The involvement of communities in restocking and sea-ranching projects could provide an important route out of poverty, as well as offer food security to many communities.

5.4. Trace metal accumulation in sediments and Holothuroids

Trace metal concentrations in coastal marine sediments in the areas sampled around Tongatapu did not exceed guideline values, with the exception of Hg that likely stems from volcanic activity in the area. It is relatively unlikely that the current sediment trace metal levels are contributing to the depletion of sea cucumber stocks in this region. Other potential sources of pollution or marine habitat degradation such as nutrient accumulation and microplastics and should be ruled out before attributing the decline of sea cucumbers entirely to fishing pressure.

One third of solid waste disposal in Tonga is burned or dumped, and some waste dumping occurs in the oceans (Dutton & Helu, 2005). There is considerable plastic pollution in mangroves, along the shoreline, and in the intertidal seagrass and back-reef environments around Tongatapu (personal obs. Tonga 2014 and 2015). The degradation of plastics in the environment, as well as the production and expulsion of small manufactured plastics has resulted in the accumulation of microplastics in the marine environment (plastic particles less than 5 mm in size (National Oceanic and Atmospheric Administration (NOAA))) (Cole et al., 2011; Wright et al., 2013). Microplastic accumulation in the oceans is a considerable problem, entering the environment via rivers, waste-water and rubbish leakage, and can then be taken up by marine organisms (Cole et al., 2011). The uptake of microplastics from sediments has been demonstrated by the sea cucumber species *Cucumaria frondosa*, *Thyonella gemmata*, *Holothuria grisea*, and *Holothuria floridana* for which feeding on microplastics has been recorded (Graham & Thompson, 2009). Persistent Organic Pollutants (POPs) are able to sorb to microplastics, and microplastics can potentially have negative effects on the organisms which consume them (Bakir et al., 2012; von Moos et al., 2012). This has been demonstrated in the mussel *Mytilus edulis* which exhibited an inflammatory response upon the uptake of microplastics (von Moos et al., 2012). Additionally, plastics can partially block the digestive system of the Laysan Albatross, and injure the gut of chicks (Fry et al., 1987). Small plastic pollution has been demonstrated in Hawaiian sediments (McDermid & McMullen, 2004) and it is possible that there are microplastics accumulating in sediments around Tongatapu due to coastal waste pollution. Other pollutants which might also contribute to sea cucumber decline include persistent organic pollutants (POPs) and organotins.

Persistent organic pollutants (POPs) are chemicals which persist in the environment and can have negative effects on both people and organisms through bioaccumulation (Fu et al., 2003). Exposure to POPs can be toxic to echinoid embryos, can cause elevated rates of mortality in Antarctic krill larvae, and have been shown to cause changes in thyroid function in the freshwater fish *Oncorhynchus kisutch* (Mayer et al., 1977; Anselmo et al., 2011; Poulsen et al., 2011). Thus it is possible that while POPs were not measured in the current study, that they are present in the marine environment around Tonga and could be accumulating in sea cucumber tissues, and/or having a negative effect on these animals.

Organotins are toxic chemicals, and their use in antifouling paints on ships began in the 1960s (Shim et al., 2000; Omae, 2003). These chemicals degrade quickly in the ocean, and are taken up by marine organisms in which they can have toxic effects (Shim et al., 2000; Omae, 2006). Organotins are toxic to marine algae, have been shown to negatively affect ascidian embryo development, and can cause growth and development retardation in mussel larvae (Walsh et al., 1985; Dixon & Prosser, 1986; Cima et al., 1996). Many years following the implementation of legislation to reduce marine organotin inputs, elevated concentrations of these compounds can still be measured in marine life (Morcillo & Porte, 1998). Thus, although organotins were not measured in the current study, it is possible that these compounds represent a source of toxicity in the environment around Tonga, particularly in heavy shipping traffic areas such as Nuku'alofa port. I would suggest that future studies investigate the levels of microplastic pollution around Tonga, the presence and quantities of organotins and POPs, and to assess if these toxicants may be contributing to decreasing sea cucumber abundance, or to the incapacity of populations to recover from fishing efforts.

In the present study, Tukumotu was the most polluted site, and there were differences in trace metal accumulation between near and far shore areas, indicating that anthropogenic sources of trace metals may be accumulating in coastal sediments. Although these levels were beneath guideline values, this research indicates that humans are influencing the trace metal concentrations of sediments at the sites studied. Mitigation of anthropogenic effects on the environment in Tonga through organic farming and improved solid waste management, is encouraging. It is likely that such steps are necessary to prevent further contamination of nearshore sediments with trace metals.

The daily consumption of As, Ni, and Zn in sea cucumber tissues (at the quantities specified in this study) by both Tongan consumers and those consuming the body wall as Beche-de-

mer, may present a human health risk due to these levels exceeding chronic oral reference dose guidelines (See Section 4.4.5.). Additionally, it appears that sea cucumber tissue trace metal concentrations did not reflect trace metal levels in the sediments. This phenomenon has been demonstrated in sea cucumbers from other studies (Xing & Chia, 1997; Denton et al., 2009). Thus, it would be beneficial to understand alternative routes (other than dietary through sediment) of trace metal uptake in sea cucumber species, and to take steps to mitigate sea cucumber exposure via these routes in Tonga.

5.5. Summary

Overfishing can lead to the extinction of species and large ecosystem-wide changes, particularly if one species is fished to extinction, and its trophic-level ‘replacement’ is then also fished (Jeremy et al., 2001). Sea cucumbers are considered to be an ecosystem engineer, creating burrows in marine sediments (Coleman & Williams, 2002). The removal of ecosystem engineers can have detrimental effects on species richness, diversity and oceanic processes (Coleman & Williams, 2002). Furthermore, overfishing removes energy from within an ecosystem, and affects the availability of energy at higher trophic levels (Libralato et al., 2008). There are also unintentional effects of fishing on ecosystems such as the destruction of habitat and changes in population size and structure (Pikitch et al., 2004). Thus, there are both community and ecosystem effects of sea cucumber overfishing.

In summary, this research was conducted with the overall aim of providing the Tonga Fisheries Division and the governing bodies of Tonga with information which may benefit the future management of the traditional and commercial export sea cucumber fisheries in Tonga. There are many more knowledge gaps to address surrounding the sustainable management and use of this resource, and I hope that the research presented through this work may provide some future management directions which may benefit Tongan communities and the marine ecosystem which is of such key socioecological importance.

6. Bibliography

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7. Appendix

7.1. Controlled Reference Material (CRM) recoveries

Table 7.1. Certified values for concentrations of each metal expressed as mass fractions (mg kg^{-1}) unless specified as %, and the average % recovery for each metal for the standard reference material (SRM 2702, Inorganics in Marine Sediment) used in ICP-MS analysis in 2014 and 2015.

Metal	Certified Value	% recovery 2014	% recovery 2015
Fe	$7.91\% \pm 0.24\%$	84	76
Co	27.76 ± 0.58	92	84
Ni	75.4 ± 1.5	70	70
Cu	117.7 ± 5.6	93	82
Zn	485.3 ± 4.2	94	83
As	45.3 ± 1.8	106	94
Cd	0.817 ± 0.011	107	103
Hg	0.4474 ± 0.007	149	133
Pb	132.8 ± 1.1	92	94

Table 7.2. Average CRM % recoveries ($\mu\text{g g}^{-1}$) for the Standard Reference Material 2976 - Mussel Tissue (Trace Elements and Methylmercury) (Mussel) and for the Standard Reference Material 1577c – Bovine liver (Bovine).

Metal	Mussel		Bovine	
	Certified Value	% recovery	Certified value	% recovery
Fe	171.0 ± 4.9	88	197.94 ± 0.65	92
Co	0.61 ± 0.02	94	0.300 ± 0.018	91
Ni	0.93 ± 0.12	73	0.045 ± 0.009	72
Cu	4.02 ± 0.33	83	275.2 ± 4.6	98
Zn	137 ± 13	97	181.1 ± 1.0	94
As	13.3 ± 1.8	124	0.020 ± 0.001	67
Cd	0.82 ± 0.16	99	0.097 ± 0.001	98
Hg	0.061 ± 0.0036	102	0.00536 ± 0.00017	169

Certified values are presented as means \pm standard errors and expressed in mg kg^{-1} dry weight and were taken from the Certificate of Analysis for SRM 2976 (mussel) and the Certificate of Analysis for SRM 1577c (bovine).

7.2. Tissue metal concentrations

Table 7.3. Average tissue trace metal concentrations ($\mu\text{g g}^{-1}$ dry weight) for samples analysed by ICPMS from Tongatapu 2014 (n = 1 – 5).*

Site	DFS	Species	Tissue	Fe	Co	Ni	Cu	Zn	As
Atata	Near	BOHMAR	B	242.5	0.1	0.5	0.4	9.0	10.0
Atata	Near	HOLATR	B	50.1	0.1	0.6	1.2	25.2	68.6
Atata	Far	STICHL	B	45.8	0.0	0.3	1.0	47.4	13.2
Kolonga	Near	BOHMAR	B	273.2	0.1	0.6	0.5	12.2	11.9
Kolonga	Far	BOHMAR	B	197.3	0.0	0.6	0.5	12.8	15.4
Kolonga	Far	HOLATR	B	31.3	0.0	0.2	0.6	23.8	43.3
Kolonga	Far	STICHL	B	55.0	0.0	0.9	1.0	21.3	7.1
Sopu	Near	BOHMAR	B	133.4	0.0	0.7	0.4	12.6	7.0
Sopu	Near	STIHOR	B	59.3	0.0	0.5	0.8	27.6	8.6
Tukutonga	Near	BOHMAR	B	287.3	0.1	0.5	0.7	9.9	7.4
Tukutonga	Near	HOLATR	B	43.5	0.0	0.2	0.8	20.6	13.7
Tukutonga	Far	HOLATR	B	27.2	0.0	0.1	0.7	27.3	33.4
Tukutonga	Near	STIHOR	B	48.0	0.1	0.3	1.1	31.3	6.1
Atata	Near	BOHMAR	G	24.6	0.1	0.1	3.9	36.6	51.9
Atata	Near	BOHVIT	G	399.6	0.1	1.8	1.1	71.5	24.5
Atata	Near	HOLATR	G	146.1	0.1	1.2	3.3	46.9	74.6
Atata	Far	STICHL	G	33.1	0.1	0.3	3.3	53.6	51.4
Kolonga	Near	BOHMAR	G	97.8	BDL	0.7	3.6	75.5	7.4
Kolonga	Far	BOHMAR	G	74.8	0.1	0.4	2.5	47.3	29.9
Kolonga	Far	HOLATR	G	26.4	0.0	0.1	2.3	24.5	58.2
Sopu	Near	BOHMAR	G	78.6	BDL	BDL	4.1	54.0	7.7
Sopu	Near	HOLHIL	G	62.4	0.1	0.2	2.3	66.2	9.7
Sopu	Near	HOLLEU	G	103.4	0.1	0.4	4.0	28.5	19.1
Sopu	Near	STIHOR	G	18.9	BDL	0.7	3.4	111.0	5.4
Tukutonga	Near	BOHMAR	G	123.9	0.1	0.5	4.4	32.0	10.0
Tukutonga	Far	HOLCOL	G	52.8	0.0	0.2	2.9	30.0	13.4
Tukutonga	Near	HOLHIL	G	101.9	0.1	0.4	3.0	86.8	11.8
Tukutonga	Near	HOLLEU	G	36.2	0.0	0.1	2.6	27.9	9.2
Atata	Near	BOHMAR	I	144.2	0.2	0.7	2.3	76.0	11.0
Atata	Near	HOLATR	I	223.4	0.3	1.0	3.0	85.6	14.7
Atata	Far	STICHL	I	100.8	0.2	0.5	2.4	41.5	14.2
Kolonga	Near	BOHMAR	I	267.0	0.1	0.9	2.5	39.9	4.2
Kolonga	Far	BOHMAR	I	341.0	0.1	1.0	1.8	40.2	5.3
Kolonga	Far	HOLATR	I	151.6	0.1	0.7	1.5	27.6	32.8
Kolonga	Far	STICHL	I	92.8	0.1	0.7	1.7	34.9	3.6
Sopu	Near	BOHMAR	I	131.7	BDL	1.3	1.6	35.4	1.4
Sopu	Near	STIHOR	I	207.0	0.2	0.7	3.1	61.1	4.8
Tukutonga	Near	BOHMAR	I	807.3	0.3	0.4	2.7	27.1	12.2
Tukutonga	Far	HOLATR	I	418.2	0.2	0.7	2.0	34.1	19.6
Tukutonga	Near	HOLATR	I	237.5	0.2	0.7	3.7	31.8	3.9
Tukutonga	Near	STIHOR	I	80.5	0.3	0.4	3.0	35.7	4.9
Atata	Near	BOHMAR	R	336.4	0.2	0.7	2.2	38.0	12.5
Atata	Near	HOLATR	R	326.0	0.3	0.8	6.4	197.6	37.5
Atata	Far	STICHL	R	133.5	0.1	0.6	1.8	28.5	14.8
Kolonga	Near	BOHMAR	R	329.9	0.2	0.5	3.0	55.5	18.7
Kolonga	Far	BOHMAR	R	281.3	0.2	0.7	2.1	61.6	17.9
Kolonga	Far	HOLATR	R	635.7	0.1	0.7	2.0	33.5	9.6
Kolonga	Far	STICHL	R	119.5	0.1	1.1	1.5	28.8	5.4
Sopu	Near	BOHMAR	R	373.5	0.1	1.3	1.8	30.1	6.5
Sopu	Near	STIHOR	R	1760.7	0.2	3.0	2.6	47.5	9.6
Tukutonga	Near	HOLATR	R	407.2	0.2	0.4	5.6	108.5	9.9
Tukutonga	Far	HOLATR	R	441.3	0.3	0.5	3.5	116.3	24.4
Tukutonga	Near	STIHOR	R	285.4	0.2	1.0	3.1	48.1	5.9

*Values with n = 1 are raw values or those which also comprised values below detection limits. Data are grouped according to site, distance from shore (DFS), species (BOHMAR = *Bohadschia marmorata*; HOLATR = *Holothuria atra*; STICHL = *Stichopus chloronotus*; STIHOR = *Stichopus horrens*; HOLHIL = *Holothuria hilla*, HOLLEU = *Holothuria leucospilota*; BOHVIT = *Bohadschia vittensis*; HOLCOL = *Holothuria coluber*), tissue (B = body wall, G = gonad, I = intestine, R = respiratory tree). Conversion factor from dry to wet weight is 0.093. Greater than 50 % Cd values were below detection limits.

7. 3. Proportion graphs displaying the results for the initial batch (n = 6) of tissues analysed by ICP-MS

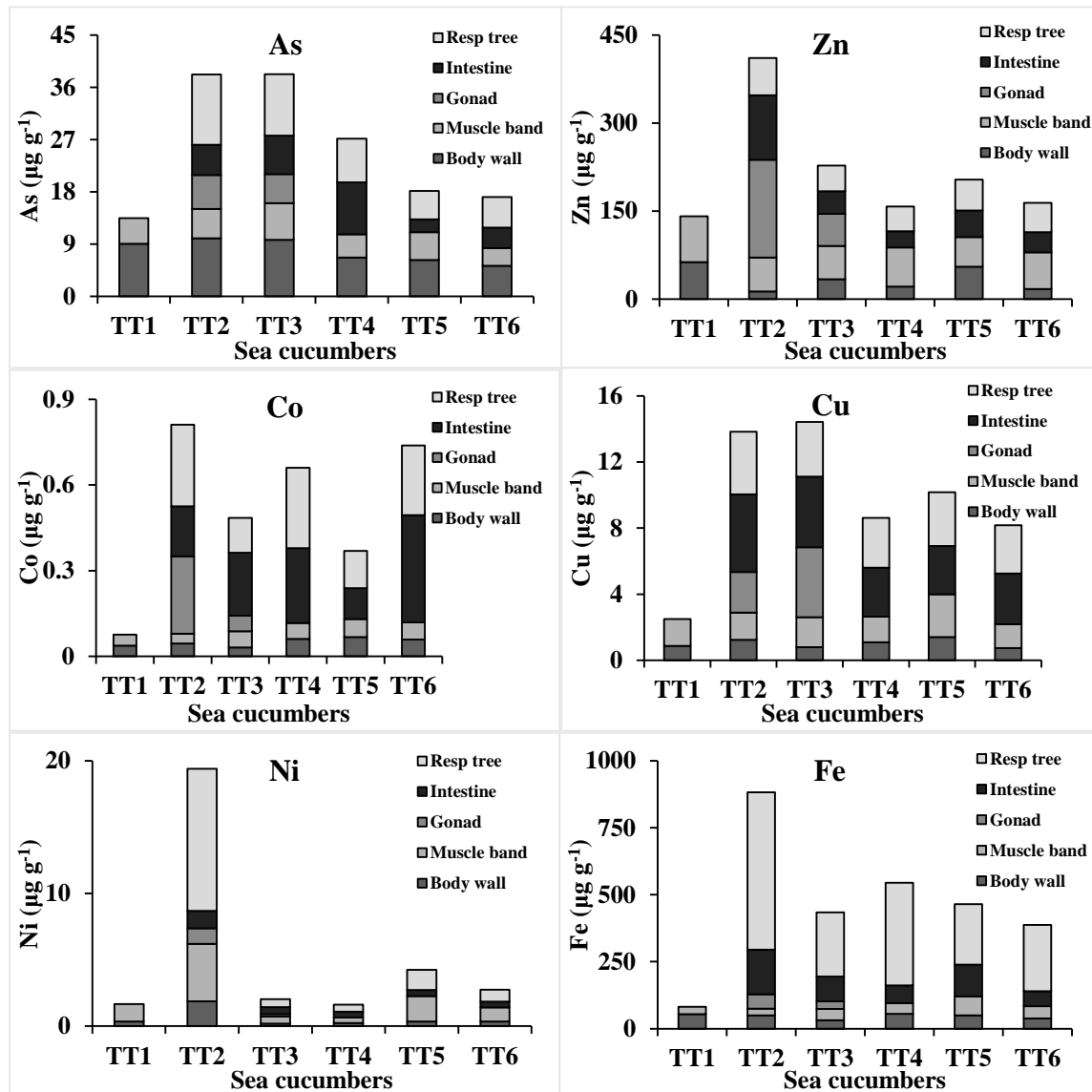


Figure 7.1. Proportion graphs for individual sea cucumbers in the initial 6 animals sampled by ICP-MS analysis for trace metal detection. The total metal concentration for each animal is presented as the cumulative metals in each tissue; respiratory tree (resp tree), intestine, gonad, muscle band, and body wall. TT1-TT3 were collected from Sopo, and TT4 – TT6 were collected from Tuketonga. All data presented is for the same species *Stichopus horrens*.