Ministry for Primary Industries
Manatū Ahu Matua

Tail fan necrosis in New Zealand red rock lobster, *Jasus edwardsii.*

Use of next generation sequencing technology to investigate potential pathogens involved in TFN and data analysis to determine the epidemiological distribution of TFN.

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Prepared for Rock Lobster Industry Council
by
Anjali Pande
Cara Brosnahan
Brian Jones
Adam Ross
Ben Phiri
Chandan Pal
Mark Bestbier

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Publications Logistics Officer
Ministry for Primary Industries
PO Box 2526
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Email: brand@mpi.govt.nz
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1 Executive Summary

This report presents the results of a Next Generation Sequencing (NGS) project looking at a small number of red rock lobster (*Jasus edwardsii*) that were either affected or unaffected by Tail Fan Necrosis (TFN).

The primary objective of this study was the use of NGS to screen for potential infectious aetiological agents of TFN. Several species of bacteria appeared to be statistically significant when examining the NGS results and comparing affected to unaffected rock lobster. However, based on a review of the literature, none of the bacterial species identified were considered likely to be a primary cause of TFN.

Additionally, catch data collected by observers were examined to generate an epidemiological picture of the distribution of TFN. Since not much data specific to occurrence of TFN exists, a data proxy was used, as discussed with members of the rock lobster industry. This data proxy was not found to sufficiently explain the epidemiology of TFN, indicating that more comprehensive and specific data needs to be collected.

Based on these results, we propose avenues for further research.

2 Introduction

New Zealand red rock lobster (or crayfish) are found around the coast of New Zealand and offshore islands. Two species are found in New Zealand in the family Palinuridae: the red rock lobster (*Jasus edwardsii)* found in the North and South Islands, Stewart Island and Chatham Islands, and the packhorse lobster (*Sagmariasus verreauxi*) found in the north of the North Island (Jeffs et al., 2013). Rock lobster are fished by customary, recreational and commercial fishers. The fishery is divided into nine Quota Management Areas around New Zealand with a commercial export value of NZ $268 million in 2015 (Seafood New Zealand, 2016).

While red rock lobster in New Zealand are relatively free of significant diseases (Booth & Kittaka 1994), one condition that causes economic loss to the red rock lobster fishery is “Tail Fan Necrosis” (TFN). TFN is a progressive chitinolytic and necrotising condition predominantly affecting the telson and uropods, especially of males, occurring at a depth of 0-30m (Porter et al., 2001; Geddes et al., 2004; Shields, 2011; Zha et al., 2018). TFN has occasionally been seen on the tail itself, but not on the carapace (D. Sykes, pers. comm.). TFN has been reported from Australia, the Caribbean, Europe and India and an aetiology has not yet been determined (Mosig et al., 2001; Porter, 2001; Mancuso et al., 2010; Leslie et al., 2012).

TFN initially presents as haemolymph filled blisters in the chitin, primarily in the telson and uropods that progress to form the characteristic blackened and eroded TFN lesions that progress and result in loss of appendages. Tails of affected lobster are therefore unmarketable. This condition does not appear to be associated with unusual mortalities.

TFN could be considered an emerging condition affecting red rock lobster and has been observed from Poverty Bay to East Cape (Freeman & McDiarmid 2009) in Quota Management Area (QMA) CRA3. TFN was first observed 5 to 8 nautical miles south of Gisborne in 1997/98 but appeared to be absent from the rest of the CRA 3 fishery at that time. In 2000, TFN was observed to be widespread from the north of Gisborne up to about 10nm south of East Cape. A few years later TFN was observed at low levels at East Cape and on the southern side of Mahia peninsula but it has not been persistent at either of these sites (G. Halley pers. comm.). Anecdotally, it has been reported that fishers are now seeing a greater number of these red rock lobster with black tails, suggesting this condition could be increasing in prevalence.

The syndrome was first investigated by Diggles et al., (2002) and at the time, studies carried out in New Zealand and Australia isolated a number of bacterial and fungal species from the blackened lesions and postulated that the blackening is due to an immune reaction of the crustacean which involves production of melanin (Zha et al., 2018). The cause of TFN remained unclear.

Previous classical techniques (e.g. bacteriology and histology) have not successfully identified an aetiological agent causing this condition. Therefore, the objective of this study was to identify a possible microbial cause(s) of TFN in New Zealand red rock lobster using NGS and comparing a small number of affected to unaffected red rock lobster.

3 Sampling and Methods

3.1 LOBSTER COLLECTION AND SAMPLING

Three affected red rock lobster were provided to the Animal Health Laboratory by the industry body. These were collected within the CRA3 fishing area using standard fishing methods. Affected lobster were selected based on the presence of lesions suggestive of TFN (i.e. blackening on the uropods, telson or pleopods and/or blistering). Lesions on each affected lobster were assessed as per the scale reported by Freeman (2017) as this is the only publicly available scale (see Table 1, taken from Freeman, 2017).

Table 1. Scale of tail fan necrosis on the lobster specimen

Score	Description
0	Necrosis absent (no obvious signs of blistering or blackened areas on any part of the lobster)
1	Necrosis present (small (< 2 × 2 cm) area of blistering or blackening on telson or uropod)
2	Necrosis present (area > 2 × 2 cm showing blistering or blackening; generally more than one uropod and/or telson affected; uropod or telson occasionally missing)
3	Necrosis present (all uropods and telson affected to considerable extent by blistering and/or blackening)
4	Necrosis present (all uropods and telson affected to a large degree; necrosis spreading into muscle tissue in the tail)

Three red rock lobster visually unaffected by TFN were purchased live from a Wellington fishmonger from stocks that were derived from the bottom of the North Island (location unknown). These red rock lobster were maintained in a seawater tank prior to purchasing. Unaffected lobster were defined as those showing no evidence of TFN on visual inspection.

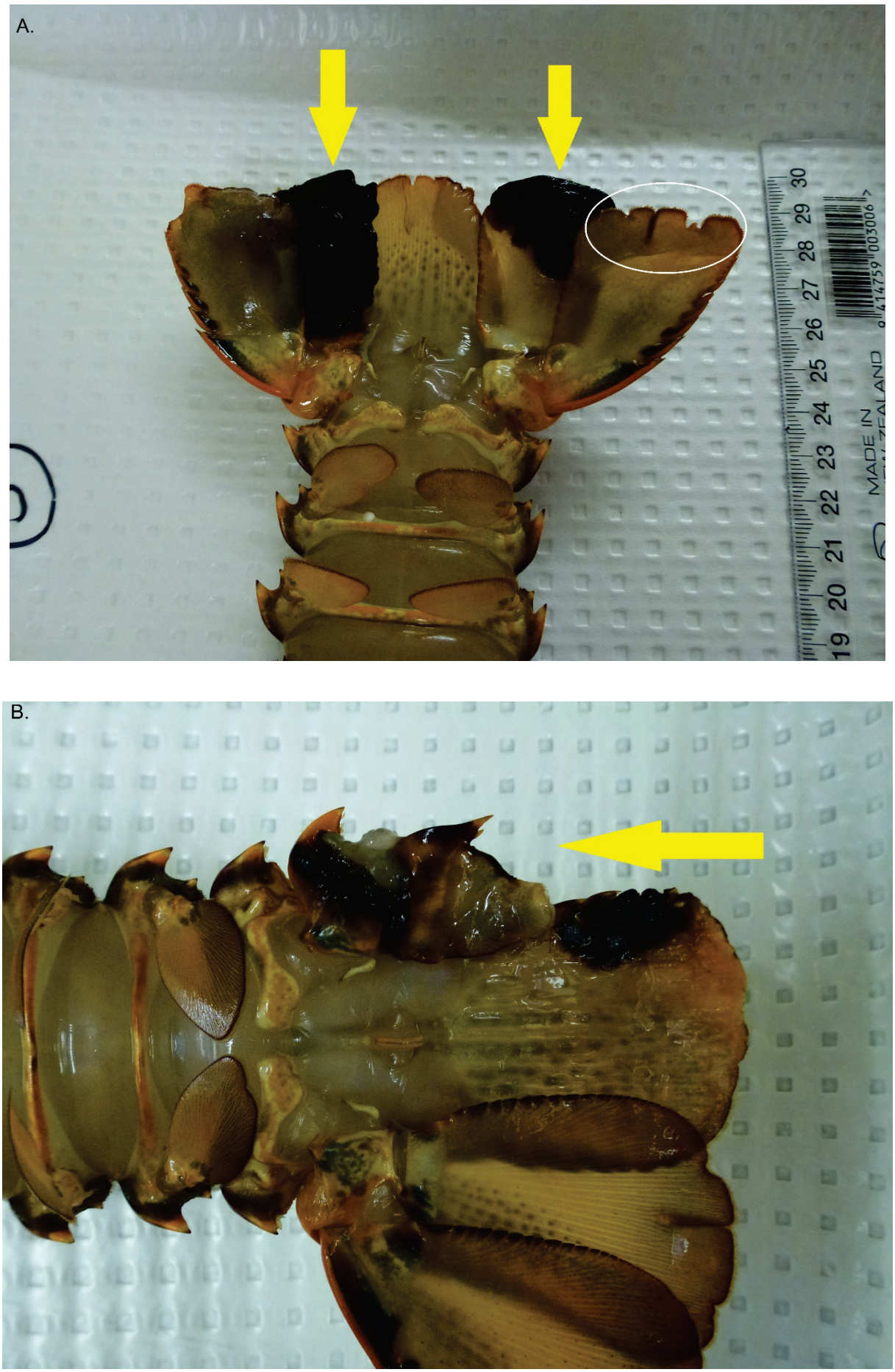

Figure 1. A. Example of an affected lobster showing blackening of uropods. Swelling at the tips of the telson and uropod is due to osmotic changes during transit of the lobster. **B.** loss of two of the uropods (yellow arrow).

3.2 BACTERIOLOGY

Bacteriology on lesions from all three affected animals was carried out immediately after the red rock lobster were euthanized. Bacteriology was not carried out on healthy animals as no lesions were present.

Swabs were taken from the leading edge of the blackened areas (Figure 1A & B) and also from the blisters as follows; the areas were swabbed with 70% ethanol, a sterile incision was made into the blisters or blackened areas and a sterile swab was inserted. This swab was then plated onto Columbia sheep blood agar (BA), tryptic soy agar + 3 % NaCl (TSA + 3%), Anacker and Ordal agar (AO) and Thiosulfate-citrate-bile salts agar.

Agar plates were then incubated at 22°C for 7 days. Common or dominant growth was purified prior to identifying to a Genus or species level using biochemical methods (Gram stain, oxidase, catalase, indole, oxidative/fermentative glucose tube test, 0/129 discs, and motility).

Sanger sequencing of the atpA gene was carried out for determination to species level on any suspect *Vibrio* sp. (Thompson et al.,2007) and the API 20E (Biomeriux, Marcy l'Etoile, France) biochemical test set was set up for non-*Vibrio* species for further identification.

3.3 RNA EXTRACTION

Tissue samples removed from euthanized lobster were taken directly into RNAlater (Ambion) as well as being stored at -80°C with no preservative added. These tissue samples were then used for assessing different RNA extraction methods to determine the optimal protocol for this tissue type based on RNA quality and quantity (See Appendix 1 for details of RNA extraction methods trialled).

Once the optimal extraction protocol was determined, RNA was extracted from tissue samples from the affected red rock lobster (n = 3) and from the unaffected red rock lobster (n = 3) and assessed for quality as above.

3.3.1 Next generation sequencing and bioinformatics analysis

A trial NGS run was carried out to ensure the RNA extracted was of sufficient quality prior to performing NGS on all samples (see Appendix 2 for more detail on the trial run).

Once it was determined that the RNA quality was satisfactory, all six samples were processed for NGS (See Appendix 3 for more details on the project run and how the data analysis was conducted).

Most abundant transcripts in both affected and unaffected lobster

Initially, the data was investigated to determine the most abundant transcripts present from both affected and unaffected samples. The abundance from each sample of the affected and unaffected lobster samples were combined to provide a total sum for affected and a total sum for unaffected lobster samples.

The data was not filtered and contained transcripts from plants, animals and other organisms that would not be considered likely to be associated with TFN.

The data was then refined to investigate only the genus that was associated with the most abundant transcripts present for both affected and unaffected lobster samples.

Transcript expression analysis

A search for possible TFN causative agents in the NGS data was conducted by comparing transcript expression between affected and unaffected lobster. It was postulated that transcripts from the possible causative agents would be more highly expressed in affected rather than unaffected lobster. To achieve this, expressed transcripts were aggregated at the genus level of the organism in both affected and unaffected lobster. This lead to the identification of genera to which possible causative agents may belong. Genus was the lowest taxonomical classification used as beyond this, current NGS outputs can be unreliable.

To minimise the effect of sequencing errors, transcripts with a group abundance score of less than 10, sequence length of transcript less than 50 base pairs, and a match of less than 99% were excluded.

Transcripts from organisms biologically unfeasible to cause disease in lobsters were excluded from the analysis. These included organisms such as plants, vertebrate mammals and other multi-cellular organisms such as insects.

3.4 ANALYSIS OF CATCH DATA

Data extracts from the Rock Lobster Catch Sampling (RLCS(A)) database were requested for the time period 1995-2017 from Quota Management Areas CRA2, CRA3, CRA4, CRA7 and CRA8.The fields of data requested are listed in Appendix 4. The database documentation can be found in Mackay and George (2002). Including data from other QMA's as well as CRA 3 was to observe if there were any indicative differences between the CRA3 data and the data from areas immediately adjacent, or those that are geographically separated (CRA 7and 8) QMA's, which could potentially explain the difference in prevalence of TFN in these areas. The *a priori* expectation is that CRA7 and 8 would be very different from CRA 3, likely due to differing environmental conditions.

RLCS (A) encompasses the catch sampling data collected by observers in the rock lobster fishery. All lobster are sexed and measured in as many pots as feasible during a day's fishing (Starr et al., 2011). This is distinct from the logbook data that the fishermen collect themselves and measure every lobster in each of 3–5 marked pots each day (Starr et al., 2011).

Anecdotal reports suggest that TFN started appearing in the fishery in the late 1990s and was commonly seen by 2000 (S. Anderson, Pers. Comm.). However, data collection is generic across management areas and there is no mechanism whereby observers could record the appearance of TFN. There is an injury index that records the number of missing appendages, including if there was tail or body damage. Whether or not a legal sized lobster was kept was also recorded (status). If a legal sized lobster was thrown back, looking at the injury index will determine whether this was due to missing appendages (three legs missing on one side is an unmarketable lobster). It was suggested by industry members that if a lobster is returned and is not listed as injured, this could be used as a proxy for the presence of TFN. This proxy is not a perfect indicator of TFN occurrence but might be useful to indicate trends and avenues for future research.

Other possible reasons for legal fish being returned to the sea include; high grading, where fishermen catch more lobster than they have quota for and retain only the high value animals (Gillis et al., 1995) and soft shell, the stage of the rock lobster directly following moult where lobster have fresh, soft shells and cannot legally be kept (Powrie & Tempero, 2009).

This data was examined to determine if there were any patterns that provide insight into risk factors that might be exacerbating TFN. This included fishing depth, time of year and the type of pot used across management areas.

Females were excluded from the analysis because the fishing season spanned the time when females are ovigerous (berried), and so would be returned to the sea. This data did not enable berried and returned females to be differentiated from non-berried and returned females and so all females were excluded from the analysis.

4 Results

The scale of tail fan necrosis in the affected lobster was as follows: Lobster 1 = scale of 2 (Fig 2A), Lobster 2 = scale of 2 (Fig 2B), Lobster 3 = scale of 2 (Fig 2C). For the unaffected lobsters, all had a scale of 0 i.e. no necrosis was observed.

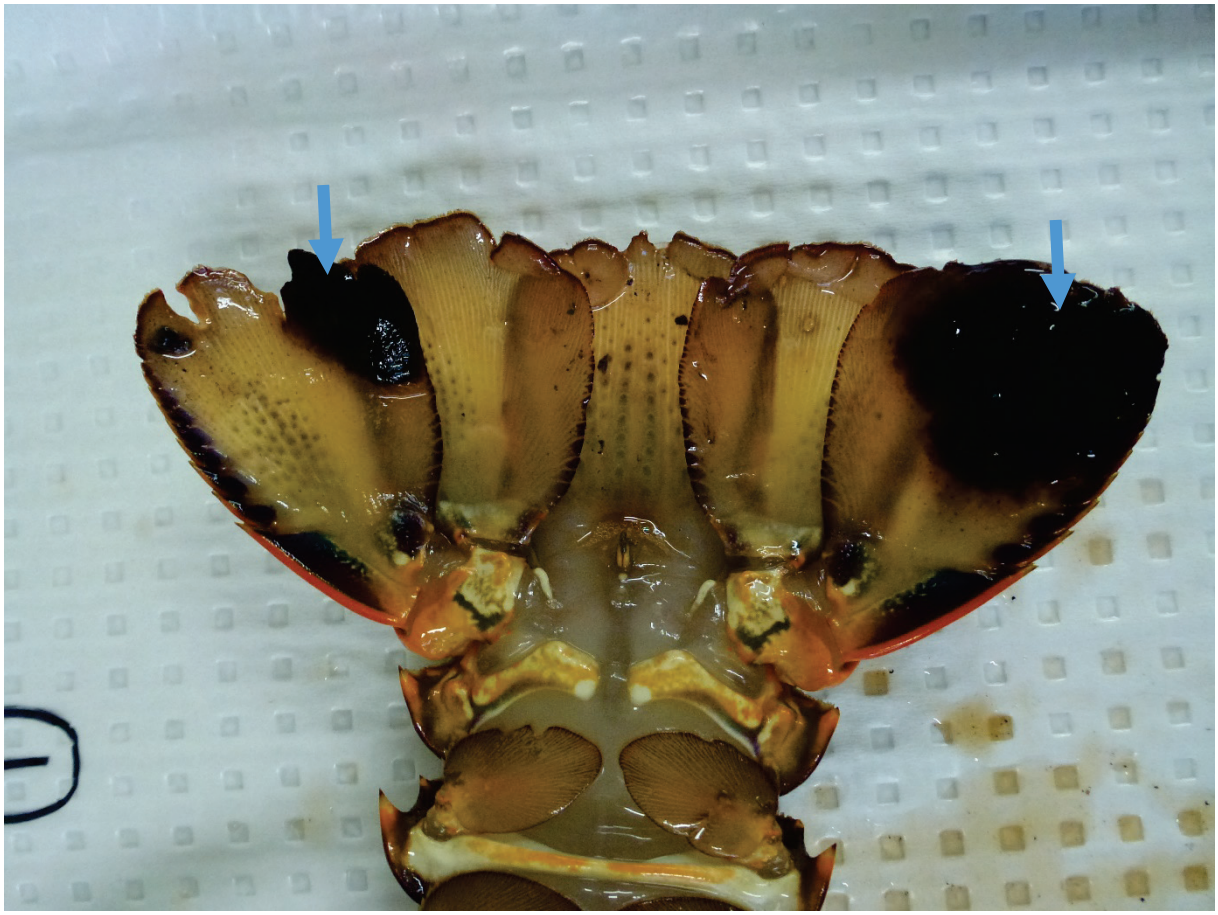

Figure 2A. Tail fan of Lobster 1 showing necrosis on the outer two uropods (arrows). TFN scale = 2 (Freeman 2017).

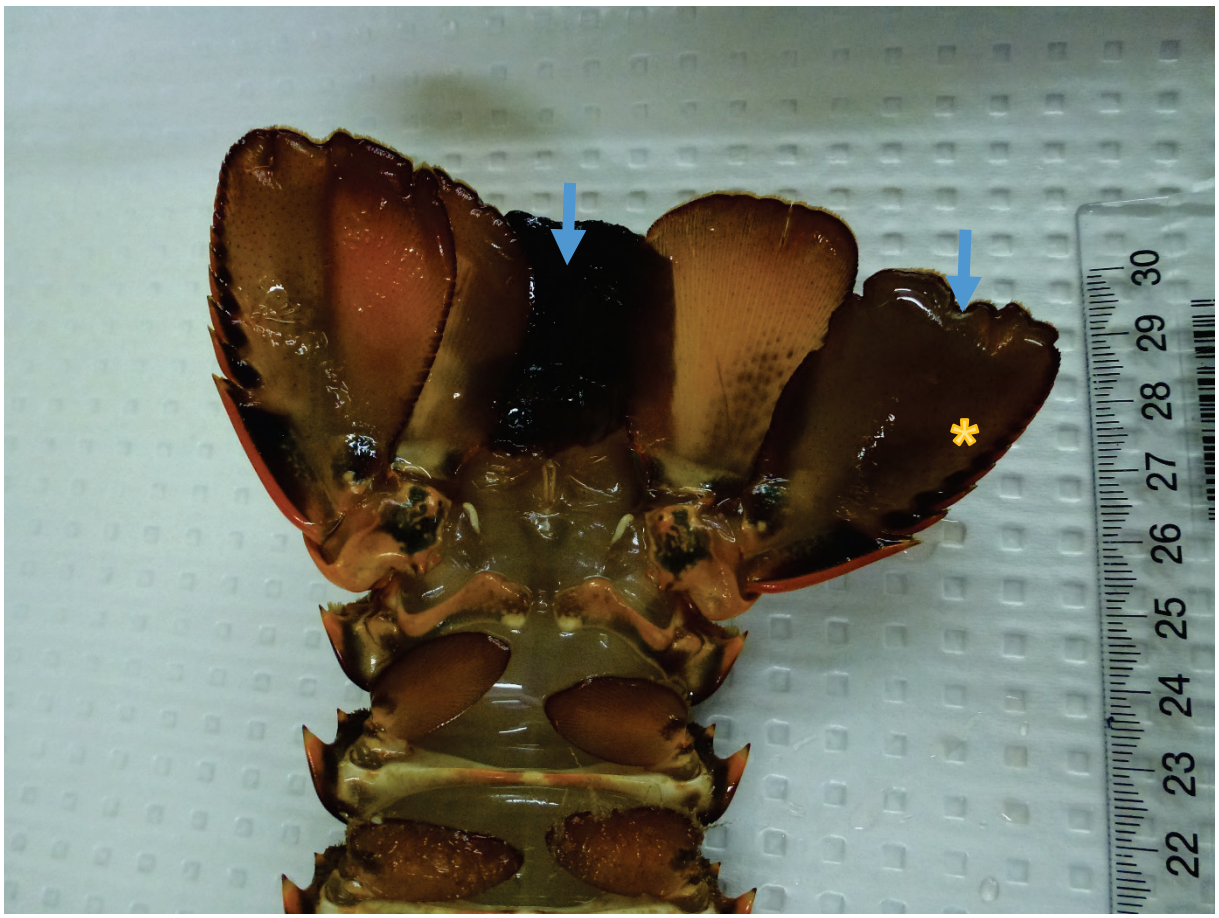

Figure 2B. Tail fan of Lobster 2 showing necrosis of the outer right-hand uropod and the telson (arrows). TFN scale = 2 (Freeman, 2017). Note the swelling of the necrotic uropod due to the osmotic changes after being removed from saltwater (asterisk).

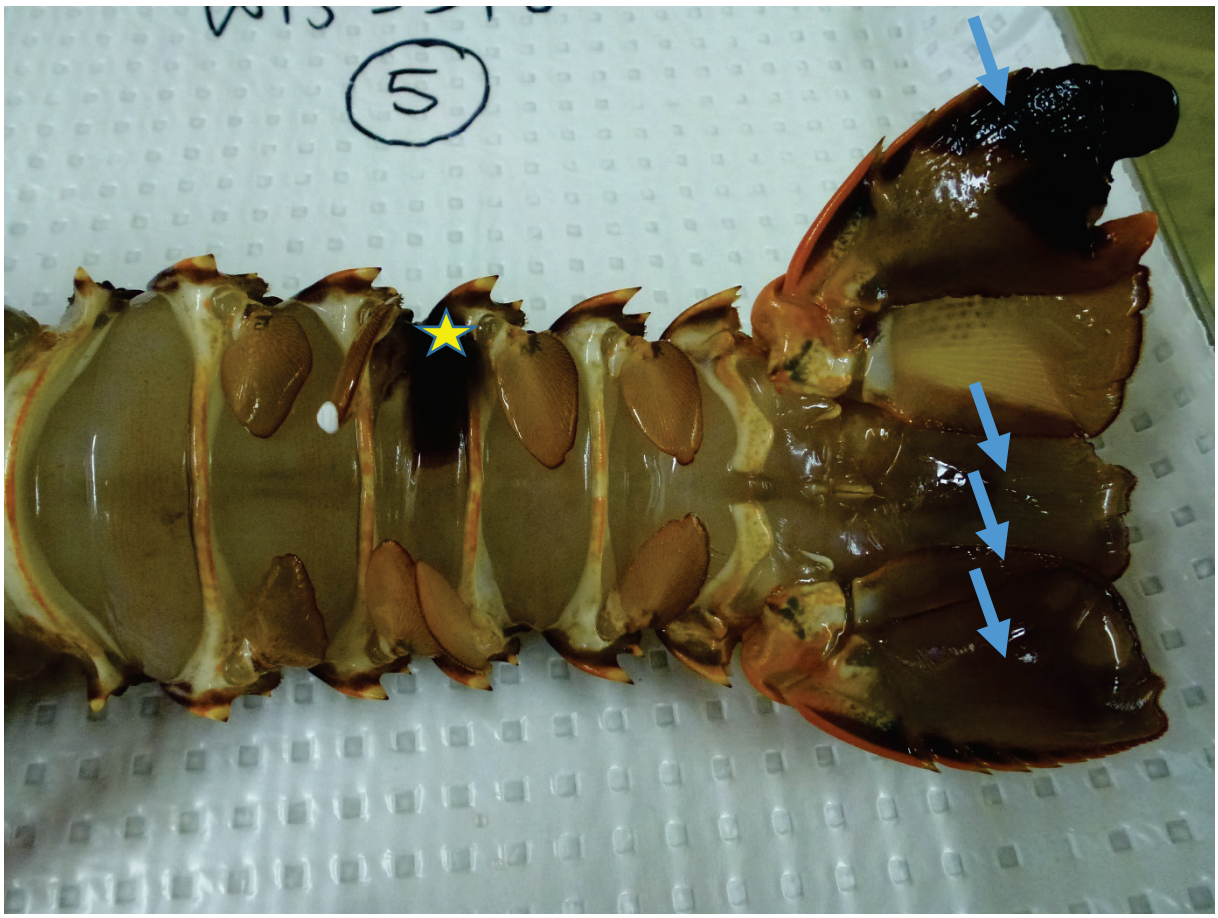

Figure 2C. Lobster 3 showing necrosis on all uropods apart from one, and the telson (arrows). Some melanisation on the abdomen (star). Note the swelling of the uropods due to osmotic changes from being removed from the saltwater.

4.1 BACTERIOLOGY

Bacteriology carried out on the blisters of affected animals revealed no bacterial growth on any agar media.

Bacteriology performed on samples taken from the leading edge of the blackened areas showed no common or dominant growth of any one species of bacteria across all six red rock lobster samples. *Vibrio splendidus* was a predominant isolate from one lobster. *Shewanella* species was isolated from four of the six lobster samples amongst light mixed bacterial growth.

Vibrio splendidus biochemical results: gram-negative rods, oxidase, catalase and indole positive, fermentative for glucose, sensitive to the vibriostatic agent 0/129, atpA sequencing showed highest similarity (99%) to *V. splendidus*)

Shewanella species biochemical results: gram-negative rods, oxidase and catalase positive, indole negative, oxidative for glucose, resistant to the vibriostatic agent 0/129, API 20E profile 060614453).

4.2 RNA EXTRACTION

Trial RNA extractions revealed that the best quality RNA was derived from tissue frozen at -80°C with no preservative and extracted using the Macherey-Nagel Nucleospin RNA plus kit (Table 2A).

RNA was shown to have acceptable concentrations (9 to 144 ng/µL) with lower concentrations of DNA. The purity of the RNA (260/280 ratio) was within acceptable limits (2.09-2.44, 2 is considered "pure" for RNA). The RNA integrity number (RIN) numbers were relatively low (2.2-

4.6, the closer the RIN is to 9 the better) however, they were deemed acceptable considering the sample type (Table 2 B and C).

Table 2A: Assessment of RNA and DNA quality for trial extraction

Sample	DNA concentration (ng µL^{-1})	RNA concentration (ng µL^{-1})	260/280 ratio	RNA integrity number
RNAlater, kit 1	14.7	14.4	2.33	1
Frozen sample, kit 1	40.4	144	2.22	2.8
RNAlater, kit 2	0.8	28.2	2.13	1.7
Frozen sample, kit 2	6.4	120	2.12	4.6
RNAlater, kit 3	3	27.2	2.55	1
Frozen sample, kit 3	1.3	44	2.42	2

Table 2 B: Assessment of RNA and DNA quality for diseased samples for main project

Sample number	DNA concentration (ng µL^{-1})	RNA concentration (ng µL^{-1})	260/280 ratio	RNA integrity number
3	0.95	37.8	2.09	2.2
4	9.34	120	2.14	2.4
6	21	138	2	2.2

Table 2 C: Assessment of RNA and DNA quality for healthy samples for main project

Sample number	DNA concentration (ng µL^{-1})	RNA concentration (ng µL^{-1})	260/280 ratio	RNA integrity number
1	1.97	52.4	2.22	4.6
2	0.25	9.36	2.28	2.3
1S	Too low	49	2.30	2.3

4.3 NEXT GENERATION SEQUENCING AND BIOINFORMATICS ANALYSIS

4.3.1 Trial run

To determine the approximate amount of ribosomal RNA sequence (rRNA) that was present, the reads were clustered into operational taxonomic units (OTUs) and then subjected to a similarity search using BLAST against the NCBI database (non-redundant). Many reads were not represented by any OTUs. This could have been because they were discarded as singletons (i.e. only a single read of its kind in a sample) or during the generation of the OTUs, as the method used is an estimate of the proportion of rRNA in the sample.

The NGS dataset from the unaffected sample contained approximately 565,000 reads
The NGS dataset from the affected sample contained approximately 650,000 reads

From these preliminary results it was concluded that there was enough non-ribosomal RNA present in the sample to proceed with the project run with the six samples.

4.3.2 Project run

The project run revealed all data passed the yield and quality specifications. Data was analysed as in Appendix 3 and a list of transcripts with the organism they closely matched to was produced. This was then used to determine the most abundant genus and candidate

pathogens. It is recognised that as the red rock lobster sampled were derived from different locations, there will inherently be differences in the microbial population present. Therefore, two analyses were conducted; most abundant transcripts and a comparison of affected vs unaffected.

Most abundant transcripts in both affected and unaffected

The non-filtered data revealed the most abundant transcripts in both affected and unaffected lobster samples showed highest similarity with bacterial species (Figure 2 and 3).

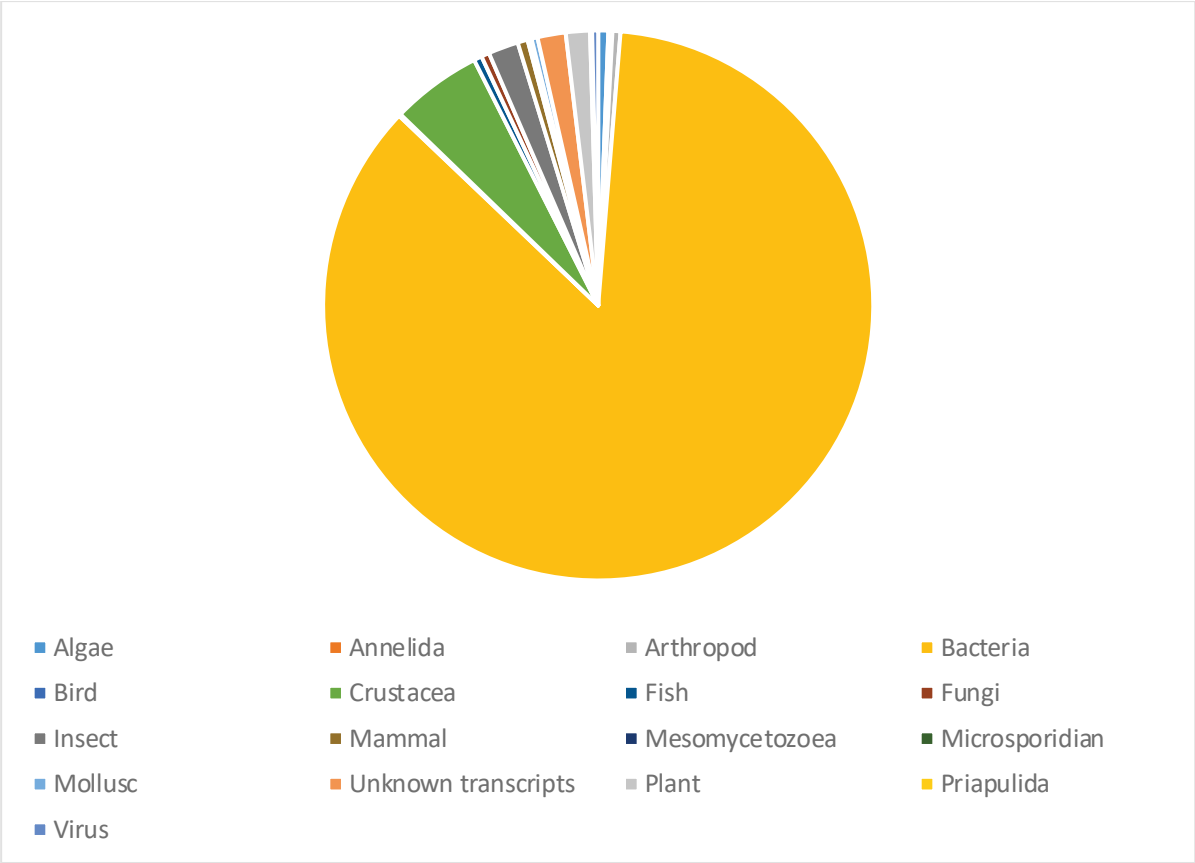

Figure 2. Graphical representation of the most abundant transcripts from affected samples.

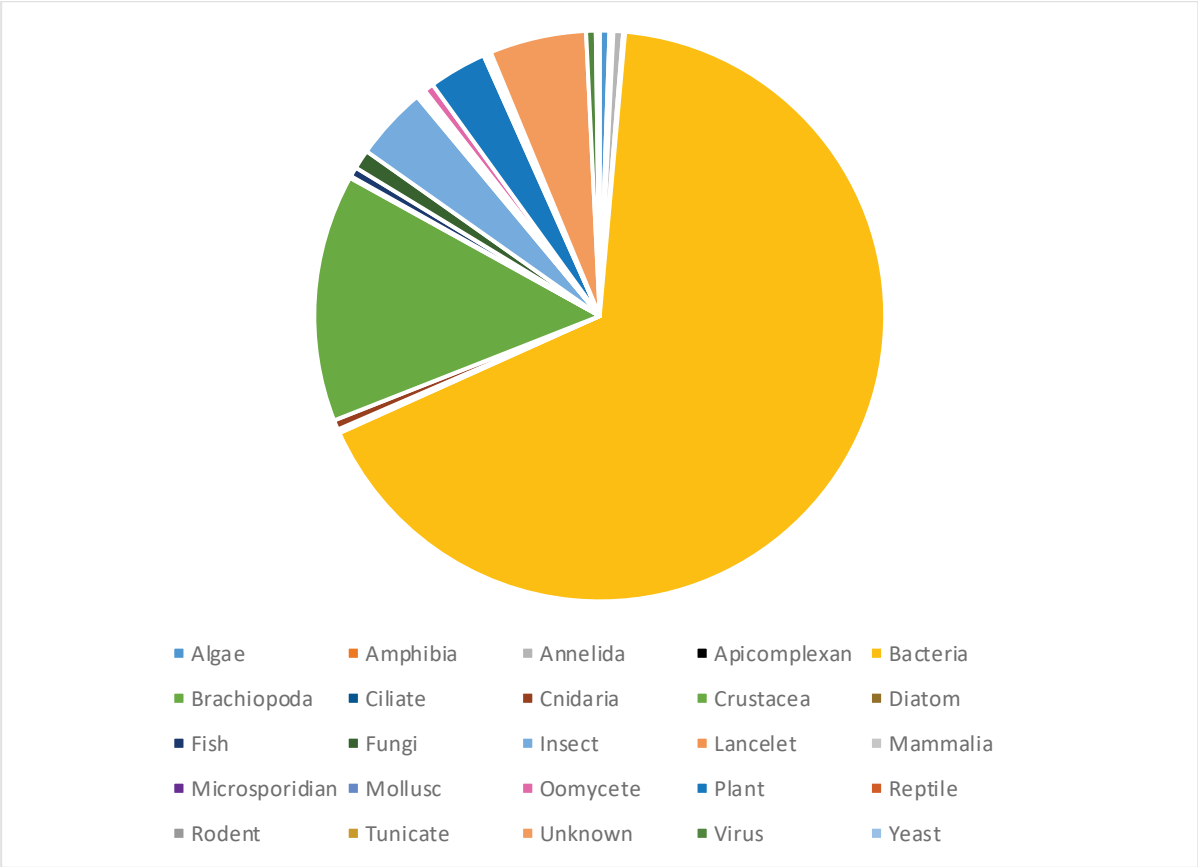

Figure 3. Graphical representation of the most abundant transcripts from unaffected samples.

The analysis of the transcripts associated with bacteria showed that there was limited diversity of bacteria and *Streptomyces* species represented 93% of the transcripts (Figure 4). In the unaffected samples, there was a high diversity of bacterial genera with uncultured bacterium representing the highest abundance at 22% (Figure 5).

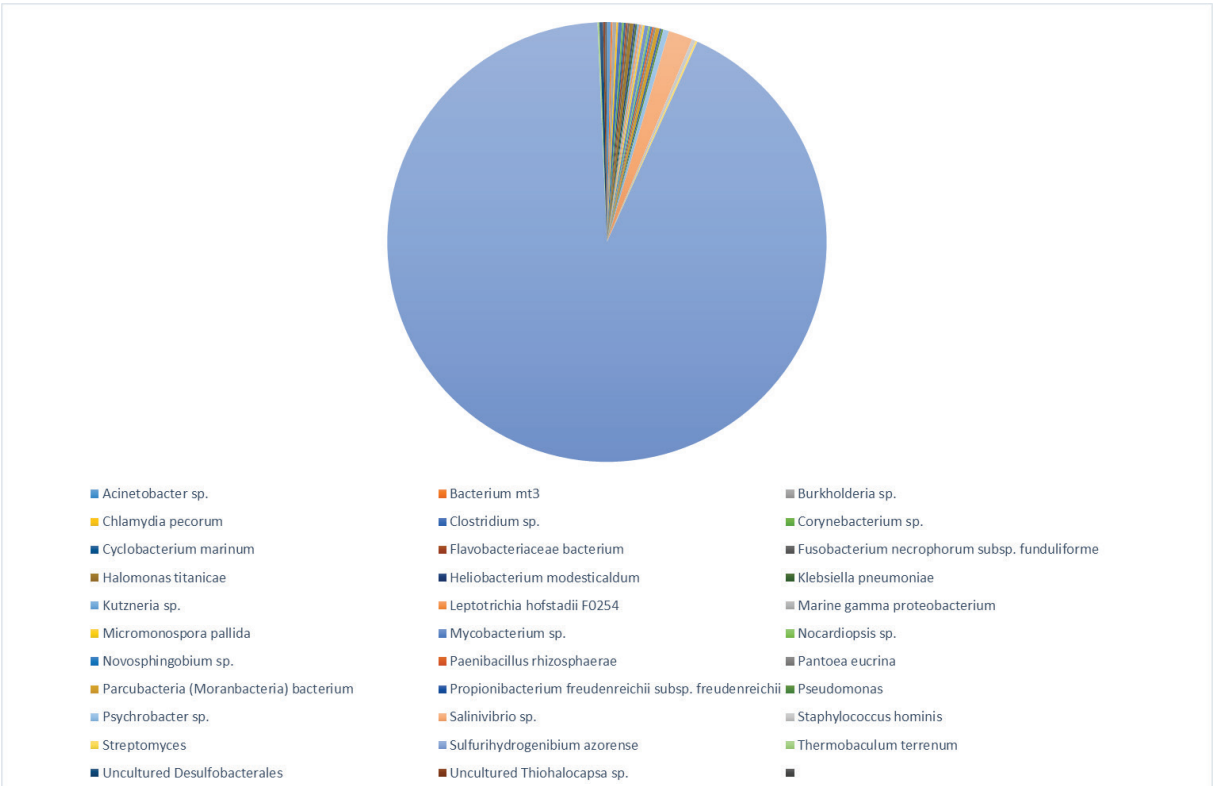

Figure 4. Graphical representation of the most abundant bacterial transcripts at a genus level from affected samples.

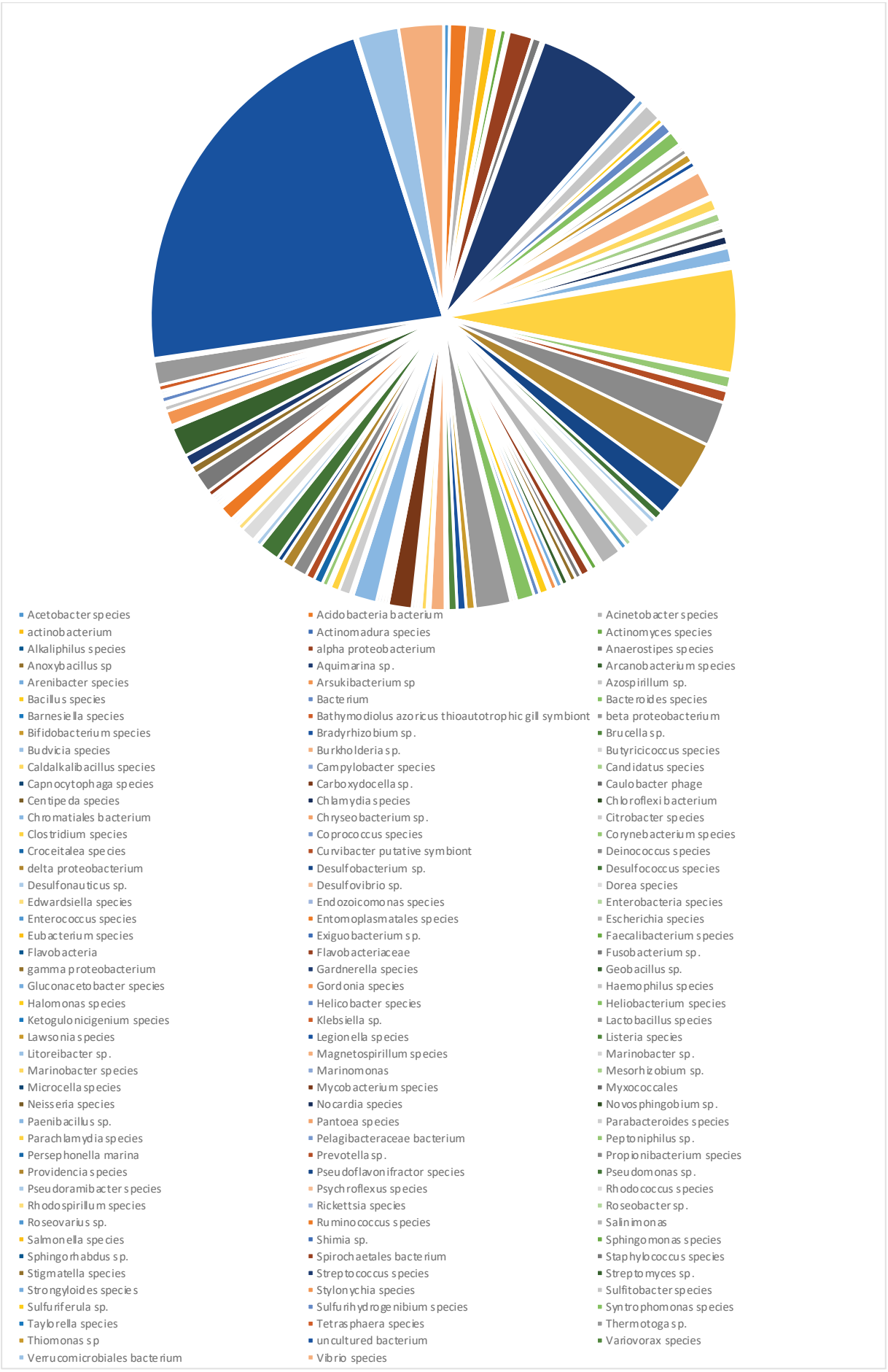

Figure 5. Graphical representation of the most abundant bacterial transcripts at a genus level from unaffected samples.

Transcript expression description and most abundant Genus

A total of 358,727 unique transcripts were derived from the six lobster samples (3 affected and 3 unaffected). Of these, 328,054 were mapped to one or more organisms by the BLAST analysis.

The organisms likely to have been the sources of transcripts that were more abundant in affected lobster than unaffected are shown in Table 3. Also shown are counts of transcripts attributable to a given organism, their abundance scores in the two groups (affected and unaffected) and the ratio of abundance scores between affected and unaffected lobster. For instance, there were two transcripts attributed to the Phylum *Actinobacteria* and their abundance score in affected lobster was 1265 and zero in unaffected lobster. Thus, there were infinitely more transcripts attributed to *Actinobacteria* in affected lobster than in non-affected ones. Similarly, 22 transcripts were attributed to the genus *Pseudoalteromonas* and their abundance in affected lobster was 82.42 times higher than in non-affected lobster.

Table 3. Organisms likely to be sources of the transcripts that were more abundant in affected than unaffected lobster, accompanied with counts of attributable transcripts, their abundance scores and abundance ratio.

		Abundance scores		
Genus	**Transcript count**	**Affected**	**Unaffected**	**Abundance ratio**
Shewanella	6	88	0	∞
Athrobacter	2	57	0	∞
Litoreibacter	1	10	0	∞
Psychrobacter	178	9427	1	9427.77
Streptomyces	1857	327445	66	4944.06
Pseudoalteromonas	22	370	4	82.42
Mycobacterium	2	19668	247	79.34
Propionibacterium	4	5147	354	14.50
Acinetobacter	1	30122	11656	2.58
Clostridium	1	21	12	1.83
Streptococcus	3	9437	5949	1.59
Pseudomonas	16	72133	46769	1.54
Methanocaldococcus	13	311154	204860	1.52

Additionally, any viruses or parasites identified in the affected lobster samples and not identified in the unaffected samples that were potential candidates were also given consideration even if they were not very abundant in the sample.

The virus identified was; *Armadillidium vulgare* iridescent virus (iridovirus) and the parasite identified was; *Enterospora canceri.*

A brief review of each of these organisms are listed below:

Actinobacteria

Actinobacteria are a phylum of bacteria found in both the terrestrial and the aquatic environment. This phylum includes the following species that were present in high abundance in the affected rock lobster; *Streptomyces* spp., *Mycobacterium* spp., *Arthrobacter* spp., *Propionibacterium* spp.

- *Streptomyces* species

Streptomyces spp. are ubiquitous in soil habitats and aquatic sediments (Brana et al., 2015). Some members of the genus produce secondary metabolites of clinical and biotechnological importance, some of which are toxic (Labeda, 2011; Tran et al., 2017). There are also species within this genus known to be chitinolytic (i.e. can break down chitin, the main component of the red rock lobster exoskeleton) (Ilangumaran et al., 2017).

The NGS analysis showed a much larger abundance of *Streptomyces* in affected lobster compared to control lobster. However, *Streptomyces* is considered a common environmental bacteria which is usually not a primary pathogenic agent. This spike in abundance could be due to differing environmental conditions where each sample was taken or secondary colonisation at the wound site. The fact that they are chitinolytic has negligible significance as nearly every *Streptomyces* spp. has the ability to do this (Kutzner 1981), and their ubiquity would have resulted in similar symptoms in lobster in other areas. They are also among the most studied bacteria as a source of antibiotics (Chater, 2016) and are very rarely associated with disease. Given this, it seems unlikely that the *Streptomyces* spp. is a primary pathogen in this syndrome.

- *Mycobacterium* species

Mycobacterium spp. are widespread in nature, particularly the aquatic environment. Some of these species (*M. marinum*, *M. fortuitum. M. shottsii* and *M. chelonae*) have been reported to cause disease in aquatic animals predominantly affecting finfish but also reported to infect shrimp (Lightner 1996). In fish, this bacteria causes chronic wasting and low to moderate level mortality in affected populations (Francis-Floyd & Yanong, 2002). In shrimp it can cause melanisation in the tissues and cuticle where the bacteria is present (Lightner 1996).

As well as culture, histology is the method required for diagnosis of *Mycobacterium* sp. in both finfish and shrimp and are clearly observable using this method combined with a Ziehl-Neelsen stain. As *Mycobacterium* sp. have not been observed under histology from red rock lobster affected with TFN (B. Jones, pers com., previous investigations), it is therefore unlikely that this genus is the primary pathogen in this syndrome, however it should not be discounted entirely.

- *Arthrobacter*

Arthrobacter is a genus commonly found in soil. As with *Streptomyces* species, this genus is chitinolytic, and has the ability to break down the bonds in chitin, the main component of the lobster exoskeleton (Lonhienne et al., 2001).

There are no reports of *Arthrobacter* species causing disease in crustaceans or fish and it is unlikely that these organisms are the primary pathogen in TFN.

- *Propionibacterium* species

Propionibacterium acnes has been detected in melanised spots in shell disease of American lobster, however the possibility of contamination in that study could not be ruled out (Quinn et al., 2012).

Propionibacterium species have been detected in the normal microbiome of fish (Givens et al., 2012), and there are no other reports of this organism causing disease. It therefore seems unlikely for this genus to be a primary causal agent.

***Shewanella* species**

Shewanella is a genus of marine bacteria with some species commonly associated with food spoilage (Gram et al., 1996).

Shewanella putrefaciens has been reported to cause septicaemia infections in farmed rabbit fish, presenting with lethargy, exophthalmia, haemorrhaging and necrosis on the body and mouth, and fin damage (Saeed et al., 1987).

Shewanella species have been found in the haemolymph of healthy blue crabs (*Callinectes sapidus*) (Shields et al., 2003), among the dominant microbial genera identified in lobster tissue (Bekaert et al., 2015) and in the normal flora of the spiny lobster (*Panulirus argus*) (Porter et al., 2001). Due to this genus being part of the normal flora of crustaceans, while it could be a secondary pathogen it is unlikely to be the primary causal agent of TFN.

As *Shewanella* are present in the marine environment and are regularly found in lobster tissue, it would be expected that it would present in NGS. The lack of association with affected lobster indicates that it is unlikely to be a cause of TFN.

Pseudomonas species

Pseudomonas species are ubiquitous, and may be involved in disease processes or act as secondary invaders of animals already compromised by other pathogens or adverse environmental conditions (Tripathy et al., 2007). *Pseudomonas* species have been isolated from lesions on the blue crab (Chistoserdov et al., 2005), but there was no confirmation that this was the agent causing the lesions.

There is some evidence to suggest that *Pseudomonas* species could be having an effect on disease processes (Tripathy et al., 2007). However, it seems more likely that it is part of the marine bacterial community present on the lobster and/or invading the affected lobster tissue as a secondary pathogen.

Psychrobacter species

*Psychrobacter s*pecies are commonly isolated from low-temperature environments e.g. polar seas, sea ice, found as normal microflora of fish as opportunistic pathogens, and as spoilage of fish in cold storage (McCarthy et al., 2013).

Psychrobacter species was identified as one of the dominant bacterial communities of lobster tails in Norway (Bekaert et al.,2015) and *Psychrobacter fulvigenes* has been identified from a marine crustacean (Romanenko et al.,2009). Due to this genus being part of the normal flora of crustaceans, while it could be a secondary pathogen it is unlikely to be the primary causal agent of TFN.

Pseudoalteromonas species

Pseudoalteromonas species are exclusively found in the marine environment. Some species of this genus have been reported as pathogens of sponges (Choudhury et al., 2015), and associated with shell disease in American lobster (Chistoserdov et al., 2005).

Additionally, some species have been associated with the microbial population of American lobsters (Bernardi et al., 2016). While this organism is a common marine inhabitant and seems to be a part of the normal flora of crustaceans, it is more likely to be a secondary pathogen and unlikely to be the primary causal agent of TFN.

Clostridium species

Clostridium species are widely distributed in soil, aquatic sediments and fish. *Clostridium botulinum* can cause disease and mortalities in fish (Cann and Taylor 1982), however this organism has also been reported from the meat of healthy crustaceans (Kautter et al., 1974) and in lobster hatcheries (Payne et al., 2007).

The abundance of *Clostridium* species in the NGS data was not markedly different between affected and unaffected lobster, suggesting this organism is not likely to be a primary pathogen in TFN but more likely normal flora in the samples.

Acinetobacter species

Acinetobacter species are common soil and water inhabitants (Doughari et al., 2011) and healthy crustaceans and molluscs have been reported to harbour this organism (Sizemore et al., 1975, Hariharan et al., 1995).

There is some evidence that it can be a secondary pathogen in blue crabs associated with adverse environmental conditions (Messick & Sindermann 1992). Although this organism was slightly higher in abundance in affected than unaffected samples, it is more likely this is a reflection of the normal flora and not a causal agent of TFN.

Litoreibacter species

The genus *Litoreibacter* contains three species isolated from the marine environment; the seashore and a sea-squirt (Kim et al., 2012). There have been no reports of this genus associated with disease. Due to this and the low abundance and transcript count in affected lobster samples, it is unlikely this organism is involved in TFN.

Streptococcus species

Streptococcus species infection in fish is a problem worldwide for cultured and wild populations (Agnew & Barnes, 2007; Yanong & Francis-Floyd, 2002).

Streptococcus iniae, *S. difficilis*, *S. milleri*, and *S. parauberis* are all examples of causal agents of disease in fish (Yanong & Francis-Floyd, 2002). The most common sign of *Streptococcus* infection in fish is haemorrhaging in various parts of the body, exophthalmia (pop-eye), and an increase in mortalities (Yanong & Francis-Floyd, 2002). There is little reported evidence of *Streptococcus* species causing disease in crustaceans (Cheng & Chen 1998, Hasson et al., 2009; Pappalardo & Boemare, 1982). The reported presentation of the disease has affected the hepatopancreas, white muscle or blood (bacteraemia). *Streptococcus phocae*, have been isolated from the gut of crustaceans as non-pathogenic flora and can be a probiotic agent against *Vibrio* species and *Aeromonas hydrophila* (Kumar & Arul 2009).

While the genus *Streptococcus* is an important pathogen of fish, it seems unlikely it is a causal agent of TFN. The way in which the infection has been reported to affect crustaceans is different to what has been seen in TFN. Additionally, all reports have demonstrated clear observation of the organism in histology, a finding that has not presented in any histology of TFN in the past (B. Jones, pers comm). It should not be discounted entirely that this pathogen is acting as a primary pathogen, however it seems unlikely that it would be the leading cause of TFN.

Methanocaldococcus species

Methanocaldococcus species are bacteria found in deep-sea hydrothermal vent fluid.

There are no reports of this genus acting as a pathogen. Due to this, the environment they are usually found in, and that the abundance of this organism was very similar in both the affected and unaffected lobster samples it is unlikely to be involved in TFN.

Armadillidium vulgare iridescent virus (iridovirus)

The family Iridoviridae consists of large DNA viruses that infect species of both poikilothermic vertebrates (fishes, amphibians and reptiles) and invertebrates (Piegu et al., 2014). *Armadillidium vulgare* iridescent virus has a wide host range of invertebrates; pill bug, nematode and Drosophila (Piegu et al., 2014; Poinar et al., 1980; Webster et al., 2016). There is no information available about this virus as a disease of aquatic animals, however other iridovirus are known pathogens of fish (red-sea bream iridovirus, gourami iridovirus) and crustaceans (Qiu et al., 2018).

Armadillidium vulgare iridescent virus was found in relatively low abundance within the NGS data, and associated only with the affected lobster samples. Consideration should therefore be given to a potential role for *Armadillidium vulgare* iridescent virus in the pathogenesis of TFN.

Enterospora canceri

Enterospora canceri is a microsporidian parasite living exclusively inside the epithelial cell nuclei of the hepatopancreas of the host *Cancer pagurus* (edible/brown crab) (Stentiford et al., 2007). Infected animals display no external signs of disease (Stentiford et al., 2007). Little is known about this species, but it could potentially be an emerging pathogen (Boakye et al., 2017). *Enterospora canceri* was found at low levels within the NGS data, however it was only found in the affected lobster samples. While it is unlikely that the pathogen here is identical to *E. canceri* as red rock lobster is a different host, it is possible it is a closely related species.

The significance of *E. canceri* in these samples is uncertain. However, the organisms were associated with TFN affected animals only and consideration should be given to a potential role for a microsporidian parasite in the pathogenesis of TFN.

4.4 ANALYSIS OF CATCH DATA

Using the data proxy method described in the methods (assuming that a non-injured legal sized lobster that is returned may be affected by TFN), showed some general trends as would be expected, such as higher percentages of returned lobster in CRA 3 than adjacent or geographically distinct areas, and a general increase in lobster returns in CRA3 from 2000 onwards. Examining factors such as fishing depths and seasonality showed some minor differences but did not appear to show any significant trends. Although the authors made some inferences from these results; upon discussion with industry members it was decided that there were too many confounding factors to have confidence in these results and so they have not been presented here.

It has been suggested that the type of pot may be having an effect on the occurrence of TFN (Freeman & MacDiarmid, 2009; Musgrove et al. 2005). As a result the pot type used in each management area was evaluated to see if the predominant pot type used in CRA3 differs from other areas. The dominant pot type was the same across CRA2, CRA3, and CRA4 with the majority of legal lobster being caught in rectangular HRC folded frame pots (50-54mm mesh size).

5 Discussion

Tail fan necrosis is a syndrome (first seen in the late 1990's) that has been affecting red rock lobster from Poverty Bay to East Cape in New Zealand and has been commonly seen since 2000. To date, no causal agent has been proven to be associated with this syndrome. Different bacterial species have been isolated from cases with TFN; *Aeromonas* species, *Vibrio* species, *Pseudomonas* species, *Shewenella* species *Photobacterium* species, *Aliivibrio* species, *Ruegeria* species and *Psuedoalteromonas* species (Zha et al., 2018, Musgrove et al., 2005).

None of these bacteria have been shown to be primary pathogens and it is considered likely that these are secondary opportunistic infections in already damaged lobster.

The use of traditional bacteriology to identify a dominant or common pathogen requires the organism to be culturable. This can be problematic as some bacteria need specialised media to grow, which may not be available, or they may be live but un-culturable leading to false negative results. Additionally, slow growing organisms can be outcompeted by other faster growing organisms in the sample resulting in potentially important organisms being overlooked.

It is not only bacteria that should be considered as a microbial causal agent in the TFN syndrome, a viral, parasitic or fungal agent could also be involved. There are no established cell lines that support the growth of a virus from crustaceans so traditional techniques cannot be used. Molecular tools such as polymerase chain reaction (PCR) and NGS are most commonly used for the detection of these organisms. Histology can also be used to visualise the effects of a virus. As viral particles are too small to visualise using light microscope, molecular tools are required to confirm the identity of any suspected virus present. This approach would also be used to identify and characterise parasites in crustaceans.

TFN may be a multifactorial syndrome associated with host factors, environmental factors and infectious agents. It should also be considered that a primary infectious process may not be involved in the development of TFN.

In the present study, NGS with RNA sequencing was used on a small sample size to allow for any bacteria, virus or parasite in the sample to be detected at genus level. It is important to note that NGS needs to be followed up with more traditional techniques to confirm the presence of any suspect organisms derived from the data. Therefore, traditional bacteriology was carried out in tandem on the affected lobster.

While some potential pathogens were identified in the comparison of the NGS data in unaffected and affected lobster in this study, there is no conclusive evidence to suggest that any of these may be the primary cause of TFN. However, an Iridovirus and the parasite *Enterospora* species may warrant further investigation.

Many of the bacterial organisms identified are common in soil or seawater environments, only becoming pathogenic under certain conditions, therefore caution needs to be taken in interpretation of these results. Additionally, the affected lobster were from a different location to the unaffected lobster. Different microbial diversity would be expected in different locations which may contribute to the differences seen in the abundance between the two different sample types. As the samples were taken from the leading edge of the lesions from the tail fan, NGS will pick up the community of bacteria that are on the outside as well as the inside of the lesion. Analysis of the haemolymph (which is considered to be sterile) of affected and unaffected lobster as well as the lesions may help to interpret these results. Since the unaffected lobster used in this study were bought from a fishmonger, the additional handling, and tank water might affect the bacterial populations. However, in some regards, this may also be considered an advantage because if the unaffected lobster were also from CRA3, there is a possibility that they would contain the same pathogens/ microbiota but simply be sub clinically affected which means that any differences between the unaffected and affected lobster would not be clearly identified.

Traditional bacteriology results did not identify any dominant organisms across all the lobster tested and S*hewenella* species was the most common organism identified. While *Shewenella* species has been isolated in other TFN cases (Zha et al., 2018) it is more likely this organism is a secondary pathogen. *Vibrio* species was isolated from one of the affected animals, and the NGS analysis did not show it to be common to all affected animals. *Vibrio* species are ubiquitous bacteria, even if sometimes pathogenic, so this result would not suggest it to be a causal pathogen. No bacterial growth was recovered from the blisters indicating they are either

sterile or have unculturable organisms present. As only a small number of blisters were cultured, no strong conclusions can be drawn from this result.

The analysis of the RLCS data highlighted the need for further investigation into TFN occurrence with data being collected specifically on the occurrence of TFN. At first glance the data analysis, using trends in the RLCS data as a proxy, supported what has been reported anecdotally in terms of presence of TFN being reported only from the CRA 3 area. However, after further consideration of the RLCS data, rock lobster industry members advised against using this proxy on its own because of the many confounding factors in the RLCS data. It was agreed that a more dedicated TFN data collection programme is required to create an accurate picture of the distribution and epidemiological pattern seen in the occurrence of TFN, and to try and start determining potential risk factors. A programme is being implemented to collect this data.

The data analysis did show that the dominant pot types are consistent across CRA2, 3 and 4. While it has been suggested that TFN occurrence could be related to pot handling damage (Freeman & MacDiarmid, 2009; Musgrove et al. 2005), this suggests that pot-type is unlikely to be a significant factor in TFN. If pot handling damage was a factor in occurrence of TFN, then one would expect to see TFN in other CRA management areas. However, TFN has not been reported in CRA 4.

The catch data analysis in this study focused primarily on the presence or absence of TFN, using an imperfect proxy to generate an understanding of what factors should be more closely examined to further elucidate distribution and prevalence of TFN. This was determined not to be a reliable enough proxy to warrant reporting the results of this analysis. This highlights the importance of the need to generate a more complete and larger data set on actual cases of TFN, including the severity scale, over a long time period. At the time this study was undertaken, there was a move to start collecting data specifically on occurrence of TFN, but these data sets were incomplete at this time.

6 Conclusions

Although no clear pathogen involvement was identified through the NGS data, further analysis of these data should be considered. Additionally, the use of a different sample type for future NGS work e.g. haemolymph, may help to differentiate the bacterial flora that is environmental or secondary growth compared to what could be considered a primary pathogen. Based on this investigation, future research directions are discussed below.

More research should be conducted to start building a better epidemiological picture and understanding of the progression of TFN. Industry members suspect that pollen and spray drift from pine plantations may be involved. Pine plantations are periodically sprayed with a copper based fungicide, so the copper entering the waterways should be considered as a potential risk factor. Knowledge of what risk factors, both anthropogenic and environmental, lobster are exposed to in CRA3, that are not present in other fishing areas might be very important in understanding how TFN progresses. Better data is required for a more definitive analysis of prevalence of TFN, especially if severity of the syndrome is also to be considered.

7 Future directions

Primary recommendation:

As TFN is causing economic losses in one of the CRA areas, and there are suggestions its distribution may be increasing, it is of great importance to identify as much as we can about this syndrome to be able to manage it. Studies so far have focussed primarily on the diagnostics of affected animals but a large scale detailed epidemiological study has not been undertaken to try and identify whether or not TFN spread is indeed an infectious process, or what risk factors are associated with progression of this syndrome. As these factors are narrowed down we may get closer to unravelling what multifactorial combination of the epidemiological triad of host, environment and pathogen (if any) is causing TFN.

Even if an aetiological agent is never identified, identifying key risk factors may allow management processes to be enacted, if the syndrome progression is affected primarily by a given factor.

The primary recommendation is to develop a case definition for TFN followed by a study design looking at the epidemiology and risk factors of TFN. This will necessarily involve a study that collects detailed data which will allow accurate analysis and therefore determination of distribution, prevalence and any identified risk factors.

Additional work recommendations:

Environmental assessment:

- More detailed analysis to determine if TFN is prevalent in any other lobster fishing regions and a paper based exercise to see if there are any different environmental conditions between the locations that could be associated with TFN. Data collected from the above recommended study may be used to do this along with existing environmental data in the first instance, but if not enough environmental data exists then this may need to be collected.
- Experimental field work in different locations within CRA 3 (using anecdotally reported hotspots vs areas where less TFN is reported) and different times of the year to assess TFN presence/ severity in healthy/damaged lobster – assessing if, and which environmental conditions might be associated with TFN. If possible, this should be repeated in non TFN affected areas as a control. No affected lobsters will be moved to areas previously not known to have TFN.
- Assess the environmental factors that are hypothesized to be involved in TFN (water chemistry, soil chemistry and makeup)

Infectious cause assessment:

To determine if an infectious agent is associated with TFN, infectivity trials could be conducted which include;

- Under controlled environmental conditions (tank trials)
    - Cohabitation studies of affected lobster with healthy/ damaged lobster to see if TFN can be transmitted by this mechanism.
    - Immersion trials with candidate pathogens suggested in this study with lobster that had both been “artificially damaged’ and not, to see if TFN develops
    - Change the environmental conditions in both of the above regimes to try and identify if there is a “perfect host/environment/ pathogen triad” that causes TFN to manifest.

Other work:

- Identify whether holding affected animals in tanks at lower water temperatures causes TFN reversal (this has been hypothesized). Is recovery possible? Conversely does increased water temperature affect TFN progression.

- How moulting affects lobster with TFN? Is recovery possible? What does this look like?

- Another NGS analysis with lobster from different areas of New Zealand - suggest looking at the haemolymph as well as the lesion tissue itself. This may help to establish a picture of what 'normal' flora looks like to identify changes in affected lobster.

- Metabolic pathway analysis with the NGS data from the present study

- Identify the sequences for the transcripts of the *Enterospora canceri* and *Armadillidium vulgare* iridescent virus (iridovirus) and determine how similar they are to existing *Enterospora* species and Iridovirus. If warranted, consider a qPCR test for these organisms based on the sequence and test a larger number of affected and unaffected red rock lobster to statistically test the strength of the correlation between the presence of the *Enterospora* species and Iridovirus with TFN affected lobster.

- Analyse/ re-analyse stock monitoring(RLCS)/ logbook data once data on actual TFN occurrence is available to:

    - Identify hotspots of TFN prevalence (leading to - what is different about these hotspots)
    - Obtain an indication of changes in prevalence over time
    - Build on the analysis conducted in this report

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Appendix 1 – extraction kit trials

Three RNA extraction kits were trialled to determine the best method for extracting RNA from lobster tissue:

- Qiagen RNeasy mini kit
- Machery Nagel Nucleospin RNA plus
- Ambion PureLink RNA mini kit

Tissue from affected lobster was trialled from tissue stored in RNAlater (R) and tissue stored at -80°C (F) to determine the best RNA extraction method. The quality of RNA was assessed using the Nanodrop, Qubit and bioanalyser to determine the optimal RNA extraction method.

Tissue (30 mg) from each storage condition was taken and ground using a mortar and pestle under liquid nitrogen. RNA was then extracted from the tissue as follows:

Kit 1: Qiagen RNeasy mini kit: Sample R1A and F1A

- Take 30 mg of RNA later fixed tissue and grind under liquid nitrogen
- Add 600 uL buffer RLT to the ground tissue sample and homogenize by passing the lysate at least 5 times through a blunt 23-gauge needle fitted to an RNase-free syringe
- Continue with step 2 of the manufacturer's protocol.
- Elute in 30 uL – repeat and store elution from each

Kit 2: Machery Nagel Nucleospin RNA plus: Sample R2A and F2A

- Take 30 mg of RNA later fixed tissue and grind under liquid nitrogen
- Transfer the suspension to a 1.5 mL screw cap microfuge tube with 350 uL LBP lysis buffer and mix immediately
- Continue with step 2 of the manufacturer's protocol
- Elute in 30 uL – repeat and store elution from each

Kit 3: Ambion PureLink RNA mini kit: Sample R3A and F3A

- Take 30 mg of RNA later fixed tissue and grind under liquid nitrogen
- Add 600 uL lysis buffer with 2-mercaptoethanol.
- Homogenize by passing the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe
- Continue with 'Binding, washing and elution of RNA' step of the manufacturer's protocol.
- Elute in 30 uL – repeat and store elution from each

Samples were then subjected to RNA extraction using the Machery Nagel kit from three heathy samples as follows:

Homogenise ~30 mg of tissue sample using mortar and pestle under liquid nitrogen
Samples:

- Lobster frozen tissue – 1, 2 (dunked in 70% ethanol, rinsed in PBS)
- Lobster frozen tissue – 1 spiked with RNA and DNA virus then extracted

Machery Nagel Nucleospin RNA plus: Sample 2M, 3M, 5M

- Take 30 mg of frozen tissue and grind under liquid nitrogen and homogenize by passing the lysate through a blunt 23-gauge needle fitted to an RNase-free syringe with 500 uL LBP
- Transfer the suspension to a 1.5 mL screw cap microfuge tube
- Continue with step 2 of the manufacturer's protocol
- Elute in 30 uL – repeat and store elution from each

And three disease samples as follows:

Homogenise ~30 mg of tissue sample using mortar and pestle under liquid nitrogen
Samples:

- Lobster frozen tissue from animals 3, 4, 6

Machery Nagel Nucleospin RNA plus: Sample 3, 4, 6

- Take 30 mg of frozen tissue and grind under liquid nitrogen and homogenize by passing the lysate through a blunt 23-gauge needle fitted to an RNase-free syringe with 500 uL LBP
- Transfer the suspension to a 1.5 mL screw cap microfuge tube
- Continue with step 2 of the manufacturer's protocol
- Elute in 30 uL – repeat and store elution from each

Appendix 2 – Trial NGS run to assess RNA quality (run on MiSeq platform)

A trial was run on two samples to determine if the RNA extracted from the lobster was adequate for NGS. Two RNA samples, one from an affected lobster and one from one unaffected lobster, were sequenced on a MiSeq sequencing platform (2x100 bp PE) at the Otago Genomics & Bioinformatics Facility at the University of Otago. To determine the amount of ribosomal RNA within the dataset, read 1 and read 2 for the two samples were clustered into OTUs. The representative sequence for each OTU was then subjected to similarity search using BLAST (http://blast.ncbi.nlm.gov/Blast.cgi) against the NCBI nr database. All OTUs that contained the term "ribosom" (for ribosomal and ribosome) were extracted, and the number of sequences per OTU summed.

The software "cutadapt" was used to search for, and remove adapters from the reads. For quality trimming of the data, SolexaQA++ suite's "DynamicTrim" and "LenthSort" programmes were used (http://solexaqa.sourceforge.net/). For each treatment group (affected and unaffected), Trinity (Grabherr et al.,2011) was used to construct a de novo transcriptome. The two transcriptomes were then separately subjected to similarity search using BLASTn against the NCBI nt database and BLASTx against the NCBI nr database. The transcriptomes of each treatment group were then annotated with the results from the BLAST run.

Appendix 3 – Project NGS run and data analysis (run on HiSeq platform)

Six RNA samples (three from unaffected and three from affected red rock lobster tissue) were submitted to New Zealand Genomics Limited (NZGL) for in-depth investigation. Library preps were carried out with RiboZero Human/Rat/Mouse, ERCCs added, and samples run on one lane of the HiSeq sequencing platform (2 x 125 bp v4) at the Otago Genomics & Bioinformatics Facility at the University of Otago. Preliminary bioinformatics analysis, including, quality trimming of data, de novo transcriptome assembly of RNAs present and characterisation of the detected transcripts was then carried out by NZGL.

Pre-processing of sequence data: 'fastq-mcf' (http://code.google.com/p/ea-utils) was used to check and remove adapters from the sequences. 'SolexaQA++'s DyanmicTrim' (Cox et al., 2010) was used to quality trim the data with a probability cut-off of p=0.01, and 'LengthSort' was used to discard reads shorter than 50 bp post trimming.

Transcriptome Assembly & Differential Expression analysis: To assemble the transcriptome, the RNA-Sequencing de novo assembly program Trinity (Grabherr et al., 2011) was used. All six samples (i.e. three unaffected and three affected) were used to generate a singular transcriptome. To obtain the transcript abundances for each sample, the Trinity script 'align_and_estimate_abundance.p1' using the abundance estimation method RSEM and the alignment method 'bowtie2' was used to align each of the samples to the transcriptome.

To obtain a matrix of counts and normalised expression, the Trinity script 'abundance_estimates_to_matrix.p1' using the abundance estimation "RSEM' was used. The differential expression between the two treatments was calculated using the Trinity script 'run_DE_analysis.p1', and the 'edgeR' method.

Taxonomic analysis: To obtain high confidence in taxonomic origin detection of the sequence data, we investigated taxa present in the transcriptomes of the unaffected and affected samples using both short reads and assembled contigs. Short reads matched to small-subunit rRNA sequences (SSU, 16S/18S) were extracted from individual transcriptomes using Metaxa2 (version 2.2, Bengtsson-Palme et al.,2015) for taxonomic assignment with default options. Taxonomic classification of the extracted SSU rRNA sequences was carried out down up to species and/or subspecies level using a combination of hidden Markov model and sequence alignments using SILVA database (Quast et al.,2013) as reference.

For assembled data, the transcripts from the transcriptome assembly were subjected to similarity search against the NCBI nr database (accessed on 20 June 2017) using the BLASTx algorithm. To speed up the BLAST searching, the transcriptome was split into four files then BLAST searched against the large NCBI nr database. For each query sequence, the 'top' hit (i.e. matched sequence present in the NCBI nr database) was identified using with highest bit score and lowest e-value and considered as the most probable result. The results of the differential expression and 'top' BLAST hits were then merged together.

Appendix 4 – Data fields which were requested as descriptors for analysis of catch data

area	Rock lobster statistical areas 901- 943, followed by subarea used by fishers in Voluntary Logbook Program.
sample_no	Unique sample no: 3 character sample no., 2 digit year, 5 char data source (since 1 Jan 98).
date_s	Date sampled.
season	1 digit code to say whether the sample took place during an open or closed season: 1=open season; 0=closed season
species	3 char species code: CRA=red rock lobster; PHC=packhorse rock lobster.
sex	1 digit sex code: 1=male; 2-9=female of varying maturity; 0 = hermaphrodite
carapace	Carapace length (mm).
tail_width	Width of tail (decimal mm) between the primary spines of the second abdominal segment.
injury	1 digit code of rock lobster injury: 0-8=number of missing appendages; 9=body or tail damage.
moult	1 digit code for moult stage: 1=hard shell; 0 = old shell; 5 = soft shell.
status	1 digit code for landing status: 0 = not kept; 1 = kept for landing.
vessel_no	Registration number of the vessel from which sampling was carried out on.
wind1_dir	Prevalent wind direction during the soak time of the pots.
swell1_dir	Direction of the prevailing swell over the soak time of the pots.
method	1 char fishing method code: 1=potting; 2=diving
pot_type	2 char code for pot type: first denotes the materials used in construction; the second denotes mesh size. Refer to Rock Lobster Catch Sampling Manual or Appendix 2 of database documentation for codes.
depth	Depth in metres that the pot/set.