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Milligan, Rosanna J. (2008) *The occurrence and behaviour of Pseudoterranova decipiens and Anisakis simplex (Nematoda) in Gadus morhua and their impacts on commercial processing*. MSc(R) thesis.

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The Occurrence and Behaviour of *Pseudoterranova*  
*decepiens* and *Anisakis simplex* (Nematoda) in  
*Gadus morhua* and their Impacts on Commercial  
Processing

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Thesis submitted for degree of Master of Science  
May 2008

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University  
of Glasgow

## Summary

A Scottish seafood processor commissioned this study because the high prevalence of parasitic nematodes in their cod fillets meant they were unable to meet the standards demanded by their retailer. The aims were to determine which species of nematode were present, and whether the detection or mortality rates could be improved during processing with a view to eliminating 'live worm' complaints by consumers.

*Anisakis simplex*, *Pseudoterranova decipiens* and *Hysterothylacium aduncum* (Anisakidae) were identified from a total of 4920 specimens. All species were more abundant within the viscera, but *H. aduncum* was absent from the musculature. In the flesh, significantly more *A. simplex* were recovered from the abdominal flaps than the fillets; there was no difference for *P. decipiens*. Type of capture vessel, sea area, somatic condition and season had no effect on the abundance of nematodes.

Of the experimental treatments trialled, light, desiccation, temperature, electrocution and modified atmosphere packing had no significant effect on either the behaviour or mortality of the nematodes. Only high hydrostatic pressure (HHP) affected the mortality of the nematodes. Only high hydrostatic pressure (HHP) affected the mortality of the *Anisakis simplex* and *Pseudoterranova decipiens*. A pressure of 400MPa for 90 seconds caused 100% mortality of *A. simplex*. Lower pressures are likely to be as effective but will require further investigation. 240MPa for three minutes was the minimum treatment required to kill 100% of *P. decipiens* after 72 hours.

Using published literature, it may be possible to reduce initial numbers of nematodes by only fishing areas that are known to have a low prevalence of parasites during a given season. Avoiding regions where the final hosts (cetaceans and pinnipeds) are known to congregate would also be beneficial.

Candling remains the only commercially viable method for detecting nematodes in fish flesh, but other techniques are in development. Of these, the most promising appear to involve electromagnetic detection and imaging spectroscopy although they are not yet ready for industrialisation.

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### **Author's Declaration**

I hereby declare that the work submitted is my own, and has been carried out by myself, with the exception of the following:

- Composition of the original project brief (Prof. Douglas Neil and Dr. Isabel Coombs).
- Preparation and sectioning of nematode specimens for light microscopy (Dr. Ian Montgomery).

This thesis has been written by myself, and is entirely my own composition except where otherwise stated.

Rosanna Milligan

## **Acknowledgements**

Thanks to Prof. Douglas Neil and Dr. Isabel Coombs at the University of Glasgow for all their help and advice throughout the project. Thanks also to Linda Wood and Steven Bowie at Macrae Food Group for their help with the organisation and coordination of various aspects of the project. I would like to thank Ágúst Vilhjálmsón at Tros for his hospitality in Iceland and for providing all the cod samples that were used in this project. Thanks to Margaret Mullin for her help with the preparation and examination of the SEM and to Dr. Ian Montgomery for preparation of the sectioned specimens. My thanks also go to Eileen Harris, Curator of Parasitic Worms at the Natural History Museum for her hospitality and assistance in identifying the nematodes. Finally, I would like to thank Dr. Philip Smith at the University Marine Biological Station Millport for his help with the statistical analysis of the results.

## **Project Brief**

The following brief was agreed by the Macrae Food Group (MFG) and Glasgow University before work began on the project, detailing the nature of the problem and the aspects that should be investigated.

## **Proposal to identify nematode worm infections and their causes in cod fillets**

### **Objectives**

- To provide complete identification of the species of nematode worms infecting cod fillets supplied to MFG from Iceland
- To map the distribution of nematode worms in the gut and other tissues of cod
- To determine the rate of migration of nematode worms into the muscle tissues, *post-mortem*, and the causes of these movements.
- To identify and test some novel methods for identifying nematode worms in cod fillets
- To recommend best practice to prevent worms appearing in the marketed product

### **Background**

MFG wishes to reduce the number of complaints from customers of their fresh cod fillets concerning the occurrence of nematode worms.

They appreciate that there is a relationship between the fish and seal populations as hosts for the parasitic worms, but that the underlying biological facts need to be clarified, since the nematodes involved may be from one or more candidate species. In fact, helminth parasites of cold-ocean fish tend to have low host-specificity, and larval sealworms have been reported from over 75 species of fish, including cod. There are different species of sealworm, and each has a different life history pattern, with different final hosts. Another worm, *Anisakis simplex*, is also known to infect cod, but has whales as a final host, rather than seals. The issue of identification of the species of worm encountered in the cod fillets supplied to MFG therefore needs to be resolved before appropriate preventative measures can be recommended.

Current methods to locate and remove nematode worms from cod fillets involve ‘candling’ using transmitted light from a light box. This is performed both before dispatch from the Icelandic supplier, and again at Fraserburgh.

Attempts to further reduce the occurrence of worms by freeze-thawing the fillets have proved relatively successful, but this causes an unacceptable deterioration in the texture of the flesh.

It is therefore intended to return to handling fresh product, but in order to improve detection and to meet a ‘zero tolerance’ standard, a more thorough understanding of the biology and behaviour of the parasite involved is sought.

### **Expertise at the University of Glasgow**

The Institute of Biomedical and Life Sciences (IBLS) at the University of Glasgow has accumulated expertise in both fish biology and in parasitic diseases within two of its research Divisions: Infection & Immunity and Environmental and Evolutionary Biology.

Nematode diseases of both humans and animals has been the focus of much research work by various staff members of these IBLS Divisions, and particular knowledge of fish parasites is brought by Dr Isabel Coombs. Research teams in IBLS led by Dr Douglas Neil also have experience in measuring quality parameters in the flesh of fish and shellfish, including flavour analysis (HPLC), texture profile analysis and sensory evaluation.

The laboratories within IBLS are equipped to perform the tasks required, including: light and electron microscopes; histological facilities; general biochemical laboratories; state-of-the-art molecular facilities (if required). For any work requiring the examination of fish carcasses, licensed procedure rooms are available. In addition, we can access the facilities of the UMBS Marine Station, Millport, including the RV Aora, which is equipped to fish for cod using various forms of commercial gear.

A highly qualified graduate student has been identified who could undertake the required work under the supervision of Drs Neil and Coombs, and is available to start immediately.

### **Work plan**

The proposed work plan comprises 6 Work Packages (WP):

#### **WP 1: Identification of the parasite**

Working with infected fillets supplied from MFG:

- The parasites will be isolated, counted and preserved
- They will be identified according to their appearance under light and scanning electron microscopy. Candidate species are various sealworms, and *Anasakis simplex*.
- Histological sections of the worms will be prepared for light and transmission electron microscopy to confirm identification
- Comparison will be made between these fish, and others supplied more locally, or caught by the RV Aora in the Clyde Sea Area
- These identifications will be confirmed by comparison with reference data sets held by the Natural History Museum, London.

#### **WP2: Distribution of worms in the host tissues**

Working with whole cod supplied from the Icelandic supplier to MFG:

- The intensity of the worm burden will be determined, and the sizes of the worms measured (as this may affect their distribution).
- The frequency distribution of parasite numbers within the fish host will be measured
- The distribution of worms in the various organs and tissue systems at varying times *post mortem* and under different conditions will be determined by dissection
- Where necessary, this will be confirmed by histological sections
- Comparison will be made of prevalence and worm distribution in line caught and day-boat caught cod, to determine whether day boats are the highest source of worms

- Comparison will be made between these fish, and others supplied more locally, or caught by the RV Aora in the Clyde Sea Area

### **WP3: Current practice**

By visiting the facilities in Iceland, current practices for preparing the fillets will be determined in terms of:

- Capture process and location, particularly in relation to depth and to seal populations
- Post-capture holding conditions (especially temperature)
- Slaughter methods
- Times between hauling, slaughtering, gutting and filleting
- A comparison of these timings between multi-day and single day boat trips
- The filleting process
- Procedures for detecting nematode worms
- Dispatch arrangements to MFG
- By visiting the factory at Fraserburgh, the further handling and examination procedures that occur there will be determined

### **WP4: Movement of the worms post mortem**

Using whole cod from the Icelandic boats, and also the fillets removed from them, the following measurements will be made either directly, or from preserved material:

- The total parasite load per fish and the intensity of infection
- The effect of fish size on parasite load
- The distribution of parasites in the various organs and tissues, particularly in the gut and in the muscle, at different times before and after gutting
- Changes in this distribution in relation to any variation in processing practices

From these measures, a profile of the migration rate of worms from the gut to the muscle will be generated.

### **WP5: Migration rate of worms**

If the worm species found locally in fish are the same as those found in Icelandic fish, then using fish caught under controlled conditions by the RV Aora in the Clyde Sea area the following parameters will be altered:

- The time from hauling to slaughter
- The time from slaughter to gutting
- The method of gutting
- The time from gutting to filleting
- Holding temperatures
- Dwell times post-portioning
- Gas flushing in different gas mixes (CO<sub>2</sub>, N<sub>2</sub>, O<sub>2</sub>).

The effect of these changes on the numbers of worms appearing in fillets will also be measured. This will provide an estimate of the rate of migration of the worms into the muscle tissue from the gut, and will identify the main factors that affect it.

### **WP6: Novel methods for detection of nematode worms in cod fillets**

Working in conjunction with other consultants to MFG, the following methods for identification and isolation of nematode worms from cod fillets can be assessed and compared.

- Visual Inspection technology, based on digital or video imaging combined with image processing
- Thermal imaging using IR thermography

This might lead to the identification of a more sensitive and convenient method for screening for nematode worms than the currently used ‘candling’ methods.

### **Deliverables**

Technical reports will be produced that will:

- Provide a full identification of the worms found in Icelandic cod fillets supplied to MFG.
- Identify the main factors in the fishing processes that cause worms to appear in the muscle fillets

- Recommend best practices during fishing and processing to prevent worms appearing in the marketed product, so that a ‘zero tolerance’ policy is satisfied

Douglas Neil

Isabel Coombs

11/10/06

## **Chapter 1: Introduction**

### **1.1 Parasites of Atlantic Cod**

Parasites can be a major problem for the seafood industry. Many taxa from both the Protozoa and Metazoa are known to parasitize commercial fish, the most common of the latter including the cestodes (tapeworms), nematodes (roundworms) and trematodes (flukes). Such parasites occur naturally in virtually all wild animals. Unfortunately, if the wild animals are then caught for human consumption, the presence of such parasites can be problematic as many species are pathogenic to humans and can be expensive and time consuming to remove.

The Atlantic cod (*Gadus morhua*) is no exception. In a recent review of the literature, Hemmingsen and MacKenzie (2001) listed 107 species of protozoan and metazoan parasites that have been reported from Atlantic cod, the vast majority of which were present in the viscera of the fish. Since only the musculature is sold commercially as a fresh product, most of these parasites will have no effect on the consumer. Seven species were reported from the muscle, including three protozoa, a myxosporean and three larval nematodes. These are listed in Table 1.

*Table 1:* List of parasitic fauna reported from the musculature of *Gadus morhua* (from Hemmingsen and MacKenzie, 2001).

<b>Major taxa</b>	<b>Species</b>
Protozoa, Microsporidia	<i>Plistophora gadi</i> Polyansky, 1955
	<i>Pleistophora</i> sp.of Drew, 1909 and Young, 1969
	Microsporidia gen. sp. of Karasev, 1984
Myxosporea	<i>Kudoa thyrsites</i> (Gilchrist, 1924)
Nematoda (larvae)	<i>Anisakis simplex</i> (Rudolphi, 1809)
	<i>Pseudoterranova decipiens</i> (Krabbe, 1878)
	<i>Hysterothylacium aduncum</i> (Rudolphi, 1802)

Of these, the most problematic to the seafood industry are the nematodes. All three recorded species-complexes belong to the family Anisakidae (order Ascaridida) and have a near worldwide distribution. As is common for cold-water parasitic

nematodes, they are not particularly host specific; for example over 23 final hosts are known for *Anisakis simplex* (Ugland *et al.*, 2004), while *Pseudoterranova decipiens* has been recorded in 79 species of paratenic fish hosts (McClelland, 2002).

## **1.2 Life Cycle and Ecology of *Pseudoterranova decipiens***

The life cycle of *P. decipiens* involves five stages and four moults and is shown in Diagram 1. The definitive hosts for *P. decipiens* are usually pinnipeds, in which the larvae grow from stage three (L3) to four (L4) and then mature. The eggs are shed in the seal's faeces where the larvae grow to stage three and hatch (Koie *et al.*, 1995). The third stage infects at least one crustacean host (mysids are particularly important hosts at this stage (Jackson *et al.*, 1997)) before being passed on to at least one (paratenic) fish or squid host (McClelland, 2002). In the case of *P. decipiens*, the fish hosts are usually benthophagous or piscivorous demersal species (e.g. gadoids) but can rarely be pelagic crustaceans or fish (Marcogliese *et al.*, 1996). This could well reflect the habitat preferences of the mysid hosts, which are suprabenthic omnivores and predators. When the fish or squid are eaten by seals, the life cycle is complete.

It is possible for unsuitable ('dead-end') hosts to become infected with this parasite. Such hosts include organisms that will never be consumed by the definitive host, thereby preventing the parasite from completing its life cycle. Such hosts may include secondary fish hosts (e.g. large Atlantic cod), seabirds (e.g. Riley, 1971) and humans (e.g. Sakanari and McKerrow, 1989) for example. The vigour of the parasites may also be diminished by serial transmissions (McClelland, 2002), and those in secondary fish hosts may be more vulnerable to the effects of the host's immune response.

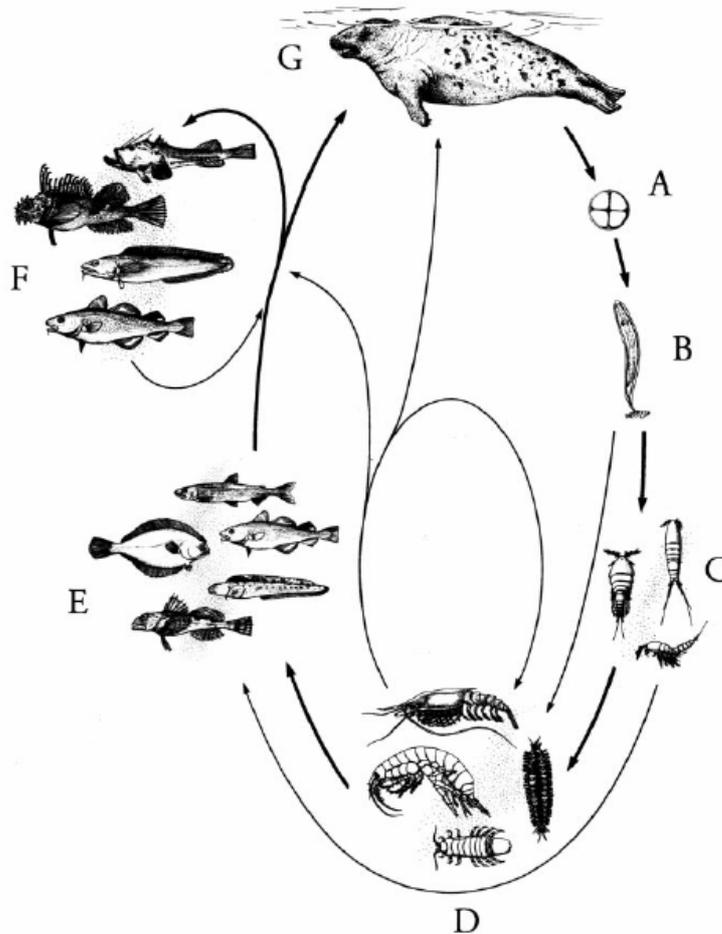


Diagram 1: Life cycle of *Pseudoterranova decipiens* showing examples of potential hosts at each stage. A. Egg; B. Free-living larvae; C. Crustacean hosts (copepods) D. Secondary crustacean hosts; D. Primary fish hosts; E. Secondary fish hosts (from McClelland, 2002).

### 1.3 The Life Cycle and Ecology of *Anisakis simplex*

The life cycle of *Anisakis simplex* is very similar to that of *Pseudoterranova decipiens*, and again involves five stages and four moults. The definitive hosts for *A. simplex* are cetaceans, and although it has occasionally been reported from pinnipeds, this has usually been at larval or L4 stage (e.g. Ólafsdóttir and Hauksson, 1998).

Larvae mature in the stomach of the cetacean, and their eggs are shed in the host's faeces. The larvae then grow to L3 before hatching (Koie *et al.*, 1995), and become infective to small crustaceans (particularly euphausiids in this case). The L3 larvae are then passed to at least one paratenic fish or squid host where they grow, but remain as L3. The fish hosts are generally believed to be pelagic species (such as

herring (*Clupea harengus*) and mackerel (*Scomber scombus*). The fish are consumed by the definitive host and the life cycle is completed.

#### **1.4 Geographic Distribution Patterns of Fish Hosts**

Studies from Norway, Sweden and Iceland have shown that *P. decipiens* is more abundant in cod from coastal regions, particularly around seal colonies and haul-outs (Apsholm *et al.*, 1995, Des Clers and Andersen, 1995, Olafsdottir and Hauksson, 1997). This is as expected, since seals are the definitive host for this parasite. However, both seals and cod can be highly mobile species, and distribution patterns can vary seasonally. Around Iceland, the highest numbers of seals are found around the west and north-west coasts (Dagbjartsson, 1973, Hauksson, 2006), and cod from these areas have a correspondingly high prevalence of *P. decipiens* (Ólafsdóttir and Hauksson, 1998).

Atlantic cod around Iceland have been described as ‘sedentary’ or ‘accurate homers’ by Robichaud and Rose (2004), meaning that they will either remain in a relatively small geographical area, or perform seasonal movements before returning to a relatively small area, for example to spawn. This can affect the overall prevalence of parasites in cod from different regions. For example, the south-west of Iceland is known to contain a major spawning ground for Atlantic cod, and cod in this region generally have lower numbers of parasitic nematodes, potentially due to the influx of ‘clean’ cod from Greenland (Platt, 1975). These trends and their significance will be discussed in more detail in Chapters 3 and 5.

By contrast, there are relatively few data on the distribution of *Anisakis simplex*. Strømnes and Andersen (2000) noted that the highest density of *A. simplex* in Norwegian waters was typically found in the upper waters of the open sea (which potentially relates to the habitat of the euphausiid hosts). Apsholm (1995) however, found that *A. simplex* prevalence in the Barents Sea was higher in ‘coastal’ areas compared to ‘offshore’ areas. Both studies note that resident populations of cetaceans in the respective study areas are likely to influence the final distribution of the parasites in these areas.

### 1.5 Distribution Patterns of Nematodes in Fish Hosts

The distribution patterns of anisakid nematodes within the fish hosts will be discussed in detail in Chapter 3. However, it is worth mentioning some of the main trends at this point. Animal hosts provide a range of microhabitats for parasites, and some preferences have been reported for *A. simplex* and *P. decipiens*.

Individual fish will naturally have different immunities and tolerances to different parasites, but this will also be influenced by the geographic location of the fish (as discussed previously), and its life history. The age and length of cod for example have been shown to correlate with prevalence and mean intensity of both *A. simplex* and *P. decipiens* in studies in both Icelandic and Scottish waters (Platt, 1975, Wooten and Waddell, 1977, Hemmingsen *et al.*, 2000, Strømnes and Andersen, 2003).

Within individual cod, *A. simplex* has been shown to be most abundant in the viscera and abdominal flaps (hypaxial muscle) of cod (e.g. Platt, 1975, Bratney and Bishop, 1992, Strømnes and Andersen, 2000), whereas *P. decipiens* is more common from the 'fillet' (epaxial muscle) portion of the flesh (Platt, 1975, Bratney and Bishop, 1992). Both species are generally reported in higher numbers from the left side of the body (e.g. Bratney and Bishop, 1992, Smith and Hemmingsen, 2003). All of these factors could be important to fish processors, as they could suggest ways of targeting cod with naturally lower numbers of parasites.

Post-capture movements of anisakid nematodes in various fish species have previously been studied in Scottish waters by Smith and Wooten (1975) and Smith (1984). These authors reported significant post-capture migration of *A. simplex* from the viscera to the muscle of mackerel and herring. Such movements have never been recorded for lean fish (such as gadoids), but could again be extremely important for commercial processors if they do occur in cod.

## 1.6 Human Health Risks

Aside from the obvious aesthetic impact and distress live nematodes could cause the consumer, there are health risks associated with the consumption of live anisakid nematodes. Anisakiasis is a gastro-intestinal disease caused mainly by *A. simplex* (and less commonly by *P. decipiens*) which can cause severe abdominal pain, nausea, vomiting and diarrhoea. Because of its similarity to other conditions the disease is often misdiagnosed, and if left untreated may become chronic with residual symptoms lasting months or years (Sakanari and McKerrow, 1989, Audicana *et al.*, 2002, Akbar and Ghosh, 2005, Chai *et al.*, 2005).

It is therefore of vital importance that cod for commercial use is treated appropriately to kill or remove any such parasites from the flesh. Anisakid nematodes can be killed thermally, either by freezing or heating the cod fillet for a period of time (Ronald, 1960, Wharton and Aalders, 2002). The EU recommends that fish be chilled to -20°C for at least 24 hours if they are to be consumed raw to ensure that any parasites in the flesh are killed (Council Directive no. 91/493/EEC). Heating to 60°C has been shown to kill *P. decipiens* within four seconds (Hauksson, 1992), and thorough cooking will therefore remove the risk of infection. However, ascaridoid nematodes are extremely allergenic and can potentially induce severe allergic reactions even when dead (Audicana *et al.*, 2002, Akbar and Ghosh, 2005, Chai *et al.*, 2005). Additionally, freezing cod fillets results in a noticeable loss of quality (in terms of texture, flavour and shelf life) as assessed by a taste panel for Macrae Food Group (Linda Wood, *pers. comm.*).

Removal of nematodes from fresh cod is currently done by ‘candling’ the fillets. This involves inspecting each fillet over a white light and manually removing any visible parasites. Candling is extremely labour-intensive (accounting for approximately 50% of production costs for Pacific cod) and inefficient (33-93% efficiency in Canada for fillets heavily infected with *P. decipiens*) (McClelland, 2002). Physical removal of the nematodes can also result in reduced yield and an inferior product (Marcogliese *et al.*, 1996). Unfortunately no alternatives have yet been identified that are effective on an industrial scale.

The aim of this study will be to confirm the identities of the parasitic nematodes in Atlantic cod from Icelandic waters, and to determine whether current commercial practices could be modified to minimise the numbers of parasites occurring in the flesh of the fish. A series of behavioural experiments will also aim to determine whether handling and storage conditions can be altered, either to encourage the nematodes to migrate out of the flesh or to kill them without seriously affecting the quality of the cod fillet. All these factors will be investigated with the ultimate goal of reducing processing effort and improving the quality of the cod fillets to a level acceptable to both commercial buyers and consumers.

## **1.7 Aims**

### *1. Determine current practice in Iceland\Fraserburgh*

To understand current practice at the Tros and Macrae Food Group factories with regard to the capture, slaughter and processing of cod, and the systems in place to detect and remove parasitic nematodes from the product.

### *2. Identification and distribution of parasitic nematodes in Icelandic cod*

To map the abundance and distribution of parasitic nematodes within the host tissues using whole cod supplied directly from Tros.

To confirm the identification of the nematodes using specimens removed by Tros and Macrae Food Group, and from samples of cod at Glasgow University.

### *3. Behaviour of parasitic nematodes under varying experimental conditions*

To determine factors that influence the movement of the nematodes following the death of the host under varying experimental conditions and using alternative commercial techniques. This will be carried out primarily at Glasgow University using infected fillets provided by Tros.

### *4. Alternative Practice*

To review published literature for alternative processing and capture methods.

## **Chapter 2: Current Practice in Fraserburgh and Iceland**

### **2.1 Macrae Food Group: Company Aim**

The Macrae Food Group (MFG) has a contract with Marks and Spencer (M&S) worth approximately £7 million, to sell portions of cod and haddock. Unfortunately, due to the high prevalence of parasitic nematodes in the cod flesh, MFG have been unable to provide fresh cod of sufficiently high quality to meet the standards demanded by M&S. M&S consider each 'worm' complaint to represent a lost customer and therefore take a 'zero-tolerance' approach to the problem. MFG want to be able to return to selling fresh, high quality cod and aim to solve the parasite problem by the end of 2007.

Using the current supply line, raw portions of fresh fish from MFG would be on supermarket shelves for approximately five days. During this time, any live nematodes left in the fillets may migrate through the fish and may be encountered by customers in-store or at home. This problem may be exacerbated by MFG's desire to provide the freshest possible product, with the result that the fish are shipped so quickly that any remaining nematodes are discovered in shops rather than in the factory. Consequently, MFG have stopped sales of fresh cod and currently only sell portions that have been frozen. While this kills the parasite and has led to a reduction in 'worm' complaints, it also lowers the quality of the fish (in terms of taste, texture and shelf life) as assessed by an in-house taste panel. The move to frozen cod has also led to an increase in complaints that the fish is 'off'. From the end of January 2007, the cod fillets will be frozen rapidly using a nitrogen freezer in Iceland.

The supply line is already in place to deliver fresh fish very quickly, and MFG are keen to resume sales, as it has required considerable investment from the company. Preventing the nematodes from entering the fillet is considered preferable to extraction, as manual extraction reduces the quality of the fillet and is a costly and time-consuming procedure.

## **2.2 Supply of fish**

All cod are supplied to MFG in Fraserburgh by an Icelandic processing company, Tros, who source wild-caught cod from a number of Icelandic fishing vessels. This has included both factory and day-boats in the past. From the 1<sup>st</sup> April 2007, the number of vessels used will be decreased to five factory boats to try and achieve greater uniformity in the final product, although day boats may still be used on occasion if no other cod are available. Full traceability systems are in place on all Icelandic vessels.

Timeline:

- Day 1 – Cod caught and gutted.
- Day 2 – Tros receive cod.
- Day 3 – Cod are filleted by Tros.
- Day 4 – MFG receive cod fillets (flown to Glasgow in 12kg boxes, via passenger flight).
- Day 9 – End of life (best before date). Competitors’ ‘end of life’ date is at least 13 days after capture.

## **2.3 Fishing Methods**

### **2.3.1 Factory boats**

The factory boats are converted pelagic (mackerel) fishing vessels crewed by approximately twenty people. A typical vessel is shown in Figure 1. They are large vessels that use demersal long-lines to catch cod and haddock and may spend up to five days at sea. To maximise the freshness of their produce, MFG only buys cod caught on the last day of any trip.

*Long-lines:* Each line has approximately 45000 hooks (Fig. 2), and around a 40% catch rate. The lines are laid out over three or four hours at a speed of approximately 10kn and are then left to ‘soak’ before being retrieved. The entire process takes approximately 12 hours. The lines are virtually always baited with frozen herring, but fresh capelin is occasionally used in February and March when in season.

*Bleeding:* As the cod are brought on board, the throat is cut to the spine (Fig. 3), and the fish die by exsanguination. This process is carried out on all Icelandic vessels, as the greatest domestic market is for dried cod. Bleeding makes the flesh much whiter

than would otherwise be possible, which is highly desirable in dried fish. As such, it is unlikely that there will be much flexibility at this stage of processing. The fish are transferred to a tank of seawater held at ambient temperature (approximately 6-8°C) for approximately 20 minutes. The water is not chilled at this stage, as the fishermen believe that this would cause the blood to clot and prevent it from draining. The water is constantly refreshed to keep the fish clean and maximise the whiteness of the flesh.

*Gutting:* The fish are removed from the tank via a conveyor belt and transferred to the gutting table. They are constantly rinsed in flowing seawater (also at ambient temperature), but may be left for up to 30mins in this environment before they are gutted. The livers are kept, and are later processed for cod liver oil or cat food. Yellow/brown worms are often found under the membrane around the liver. The remaining viscera are discarded.

*Storage:* The gutted fish are transferred into chilled water (2-4°C) before they are packed into boxes on chipped ice, using at least 40kg ice for a maximum of 300kg fish (Fig. 4).

### **2.3.2 Day boats**

These are smaller vessels, which employ 2-5 crew and typically fish closer to shore (Fig. 5). The catch is not gutted at sea, and is sold as whole (bled) fish. If this delay allows nematodes to migrate from the viscera to the flesh, this could exacerbate the parasite problem.

These boats apparently move further north in the summer to follow the cod as they move into cooler water but this may take them into areas with a higher prevalence of nematodes. However, based on descriptions of the migration patterns of cod stocks in the southwest of Iceland (Robichaud and Rose, 2004) and data on the prevalence of nematodes in cod around Iceland (Guðmundsson *et al.*, 2006), it is unclear how beneficial or even necessary this practice is.



*Figure 1: Forward section of the factory vessel Valdimar*



*Figure 2: Long-line aboard the Valdimar*



*Figure 3: Photograph of a cod after bleeding. The throat is cut to the spine.*



*Figure 4: Ice boxes for storage of cod after capture aboard the Valdimar*



*Figure 5: A typical dayboat. Gisli Sursson pictured.*

## 2.4 Processing by Tros, Iceland

Fish are received from the boats on ice, and are graded before being transferred into ice water (Fig. 6). The cod are usually supplied gutted (which is either done on the factory boats or in a separate gutting facility). The heads are removed, and the remainder of the fish is filleted (Fig. 7). The fish are then skinned, trimmed and candled. The pin bones and the abdominal flaps are removed during trimming. Each fillet is examined at two candling stations where any visible nematodes are removed by hand (Figs. 8 and 9). A sample of the nematodes removed from the fillets is counted by Tros to record the number of nematodes per kg of fillet.



*Figure 6:* Cod are transferred to ice water after grading at Tros.



*Figure 7:* Untrimmed, unskinned side of cod after filleting.



*Figure 8:* Candling a skinned, trimmed fillet. Any visible nematodes are removed at this stage.



*Figure 9:* Candling stations at Tros.

## **2.5 Processing by Macrae Food Group, Scotland**

The cod fillets are stored below 5°C at the factory in Fraserburgh. This is more controlled than in Iceland as the facilities there are not refrigerated, and fish may go through temperature spikes of up to 10°C.

All fillets are examined twice at candling tables: once on arrival, and once after portioning. Trials of a novel video detection system are also underway, which is intended to be used in addition to candling.

The cod portions are then packed for sale to M&S. Packs usually contain two similar portions, from two different fish (e.g. two tail sections, or two head sections). Packs are heat wrapped and shrunk, although this does not affect the temperature of the product.

On average, approximately 15 nematodes are reportedly removed from around 400kg of cod per day during the winter. This number is apparently much higher in the summer.

## **2.6 Chapter 2 Summary**

The Macrae Food Group (MFG) had a contract with Marks and Spencer (M&S) worth approximately £7 million to sell portions of cod and haddock. Due to the presence of parasitic nematodes in the cod flesh however, MFG could not provide fresh cod portions as live nematodes are distressing when found by the consumer. Consequently, they changed to only selling frozen fillets. Freezing kills any remaining nematodes in the fillet, but also reduces the quality of the product. MFG are keen to resume sales of fresh, unfrozen cod.

The cod used by MFG were caught around Iceland by long-line vessels (either day-boats or factory boats). All fish were killed by exsanguination and the blood allowed to drain before being gutted (factory boats only) and stored on ice. Cod from dayboats were gutted on land in a separate facility. The fish were then transferred on ice to the processing facility run by 'Tros' where they were graded, filleted, skinned, trimmed and candled. Candling involved the manual examination of the fillet over a light box and removal of any visible nematodes. This stage is carried out twice at Tros.

The fresh cod fillets arrived at MFG approximately four days after capture where they were candled again (twice), portioned and packaged for sale. The efficiency of the supply line allowed MFG to supply a product that was fresher than competitor's products. However, as long as the nematodes were present in high numbers, no fresh cod could be sold.

## **Chapter 3: Identification and Distribution of Parasites**

### **3.1 Introduction**

The only parasitic nematodes previously recorded from the muscle of Atlantic cod belong to the family Anisakidae (see Hemmingsen & MacKenzie, 2001), and it is therefore likely that this is the group found in the cod from Iceland. Although several other taxa may be present in the viscera, these are unlikely to be relevant to the seafood industry as they are unlikely to be present in the commercial product.

Those parasites recorded from the musculature include the L3 stages of *Pseudoterranova decipiens* and *Anisakis simplex*. These species are mainly found in the viscera, but will also encapsulate within the musculature (Platt, 1975). Another anisakid, *Hysterothylacium aduncum*, has been recorded as both larval (L3 and L4) and adult stages within the viscera, and as larvae within the musculature (Hemmingsen & MacKenzie, 2001), although it would be unusual to find L4 outside the alimentary canal.

Several sources also list *Contracaecum osculatum* as occurring in cod. These have a very similar internal morphology to *H. aduncum* and distinguishing between the species can be difficult. Additionally, the phylogeny of the genus *Contracaecum* is currently unclear. Previous work has suggested that the genus *Contracaecum* be split, with '*Contracaecum*' reserved for those species with piscivorous birds as the final hosts. The genera *Phocascaris* and *Hysterothylacium* would then be expanded to include those species with either phocid seals or otariid seals and birds as the respective final hosts (Berland, 1964, in Nadler *et al.*, 2000). However, more recent molecular work has challenged this suggestion (e.g. Nadler *et al.*, 2000) and has identified three subspecies of *C. osculatum*, all of which are present in seals around Iceland (Nascetti *et al.*, 1993). Nonetheless, advice from the Natural History Museum (Eileen Harris, *pers. comm.*) suggests that *C. osculatum* is unlikely to infect Atlantic cod, and that any similar specimens present are most likely to be *H. aduncum*. It will therefore be assumed that *C. osculatum* is not present in the Icelandic cod samples, although this should be viewed cautiously.

Previous studies on the distribution of nematodes in cod have shown that *A. simplex* (L3) is mostly found in the viscera, and occasionally in the muscle (e.g. Platt, 1975, Brattey and Bishop, 1992). *A. simplex* is more commonly found in the abdominal flaps than in the rest of the musculature (Strømnes and Andersen, 2000), but this trend is less clear for *P. decipiens* (e.g. Platt, 1975, Brattey and Bishop, 1992). More nematodes in general are reportedly found in the left side of cod (Smith and Hemmingsen, 2003), and it has been suggested that this may be related to the position of the liver on the left of the body. If this is the case, it may be possible to choose the least infected regions of the fillet for sale.

The prevalence (percentage of infected cod in the entire sample) and mean intensity (mean number of nematodes within an infected host) of *A. simplex* and *P. decipiens* generally increase with host age and length (Platt, 1975, Hemmingsen *et al.*, 2000, Strømnes and Andersen, 2003), which may reflect dietary or behavioural changes in the cod as they age. Factors such as the length and age of the cod may be therefore important in predicting the potential parasite load.

It is also possible that other factors may affect the prevalence of nematodes in Icelandic cod stocks such as the type of fishing vessel, fishing region, time of year or the condition of individual fish. Differences in the handling of the fish on different vessels could influence the final distribution of the nematodes, for example if they are given time to migrate from the viscera to the flesh. Smith and Wooten (1975) and Smith (1984) have shown that this can occur in 'fatty' fish species, such as herring (*Clupea harengus* Linnaeus, 1758) and mackerel (*Scomber scombrus* Linnaeus, 1758), but has never been demonstrated in 'lean' whitefish (e.g. gadoids).

The aim in this chapter is to determine which species of nematode are present in the cod being shipped from Iceland, their distribution through the cod and the effects of external factors such as capture vessel, season and location of capture. The results will be used to guide further experimental work.

### 3.2 Study Area

All cod used in this study were caught by commercial long-liners fishing around the Icelandic coast. Icelandic waters are divided into numbered zones according to a grid system used by the Icelandic coastguard, and every fish used in this study can be traced back to one of these zones (Fig. 10).



Figure 10: Map showing Icelandic waters, divided into numbered zones as used by the Icelandic coastguard. The highlighted zones show where the cod were sampled.

Attempts were made to restrict the sampling area to zones 422-424 and 472-474, to minimise the variation between fish. This was not always possible however, and the sample location was often dependent on variables such as the weather or quota considerations which could not be controlled.

### 3.3 Supply of Cod

The fishing vessels that supplied the cod samples were restricted to those vessels used commercially by MFG. These included approximately ten dayboats, and two factory boats (Table 2). The differences between these types of boat are described in Chapter 1. The dayboats provided samples of whole and gutted cod and fillets, while the factory boats provided only gutted cod.

During normal processing, batches of cod fillets would be candled by both Tros and MFG, and any visible nematodes removed. Whenever possible, the nematodes removed during these operations were collected and sent to Glasgow University for identification and analysis.

*Table 2:* Name and type of vessels used in this study, the type of samples each provided, and the identification codes of the specific samples.

<b>Vessel Name</b>	<b>Vessel Type</b>	<b>Area</b>	<b>Contents</b>
Valdimar	Factory boat	474	Nematodes
Thomas Thorvaldsson	Factory boat	474, 424, 512, 512/462, 526	Gutted cod
Sturla	Dayboat	322	Nematodes
Gisli Surrsson	Dayboat	372, 372/322, 618	Whole cod, fillets
Faxi	Dayboat	423/422	Whole cod
Hafborg	Dayboat	423	Whole cod
Agust	Dayboat	322	Nematodes
Duddi Gisla	Dayboat	322	Nematodes
Beta	Dayboat	423	Whole cod, fillets
Von Gk	Dayboat	373, 618	Gutted cod, fillets
Dogg	Dayboat	463	Gutted cod, fillets
(Unidentified)	Dayboat	422/373, 473	Whole cod

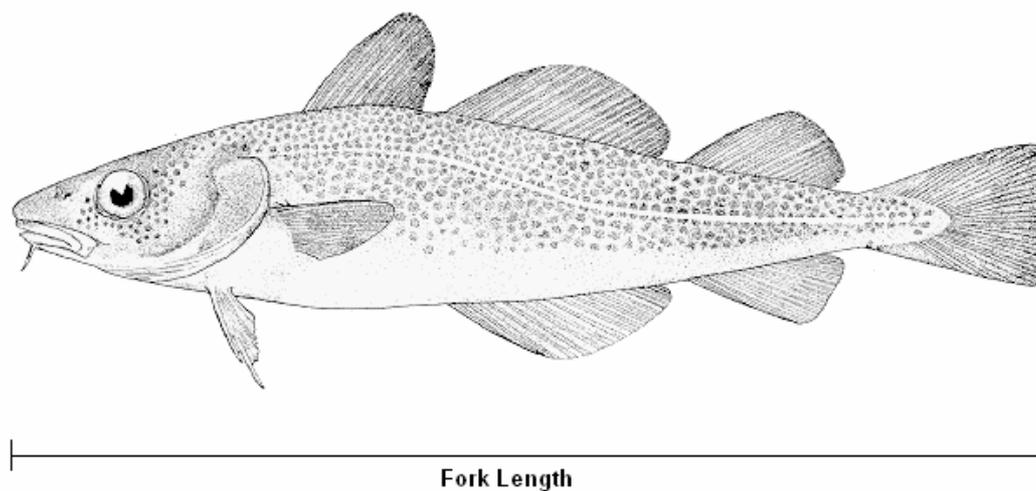
MFG stopped buying cod from dayboats from the 1<sup>st</sup> April 2007. However, since this was the only source of ungutted Icelandic cod available, these vessels continued to supply samples of whole cod for the duration of the study.

### 3.4 Methods

#### 3.4.1 Cod Dissection

The identities and distribution of nematodes in Icelandic cod was described from 21 ungutted and 24 gutted cod. The cod were stored at approximately 2°C during transportation to the UK and prior to collection and transfer to Glasgow University. Due to the lack of any suitable chilled rooms, all fish were put on ice immediately on arrival at the university which maintained the temperature at approximately 1°C for 1-2 hours until they were examined.

The fork length of each fish was recorded to the nearest millimetre by measuring from the tip of the snout to the middle of the fork in the tail (Fig. 11).



*Figure 11:* Diagram showing the fork length of a cod. Adapted from McAllister, 1990.

The fish were then dissected and examined for nematodes. The viscera (Fig. 12) were removed and the external surfaces of the organs were examined under a desk lamp. The sex of the fish was recorded at this stage. Organs with a large surface area (particularly the pyloric caeca) were carefully and repeatedly examined until no more nematodes could be found. Any nematodes removed were temporarily stored in vials containing a little distilled water to prevent desiccation. Nematodes were stored according to the tissue they were removed from. Finally, the stomach was dissected

and any contents removed. These were fixed separately in 70% ethanol. The sagittal otoliths were also removed and stored, but were not examined due to time constraints.



*Figure 12:* Dissection showing male *Gadus morhua* viscera: A. Liver; B. Stomach; C. Pyloric caeca (partially concealed by stomach); D. Large intestine; E. Gonad (testes); F. Swimbladder; G. Gall bladder; H. Spleen.

The fish were filleted based on the procedures demonstrated at Tros. The abdominal flaps were removed from each fillet (Fig. 13) and each side of the fish was kept separate. The fillets and abdominal flaps were skinned and any dark membranes removed, and were then viewed over a lightbox to replicate the candling procedures used by both Tros and MFG. However, to ensure that most nematodes were seen and removed, the fillets were also cut lengthways into strips approximately 5-10mm wide to make any remaining nematodes easier to detect. This obviously increased the risk of damaging the nematodes, but was necessary to allow detection of deeply-embedded individuals. The remaining central part of the carcass containing the spine was then candled to ensure no nematodes had been overlooked. The viscera, fillets, abdominal flaps and the head and central section of the carcass were weighed to the nearest 0.1g.



*Figure 13:* Photograph showing the separated ‘fillet’ (above) and ‘abdominal flap’ (below) from the left side of a cod.

### **3.4.2 Pepsin-HCl Digest**

To determine the efficiency of this method in detecting nematodes, a sub-sample of 7 frozen cod sides were candled, sliced and then digested using a pepsin-hydrochloric acid (HCl) digest (as described by Jackson *et al.*, 1981). Because the procedure is time consuming, frozen fillets were used as this prevented any migration of the nematodes as well as preserving the cod itself. Although the frozen nematode larvae were dead, they were not digested during the procedure (as tested by seeding specimens into the digest).

Each side was cut into three sections: the abdominal flap, the ‘loin’ (anterior fillet, forward of the anus) and the ‘tail’ (posterior fillet, behind the anus). Each section was then added to a beaker containing 750ml 0.85% NaCl saline and 15g of pepsin powder. This solution was placed on a magnetic hot plate, heated to approximately 36°C and stirred for 15mins. The solution was then adjusted to approximately pH 2.0 by adding 37% HCl, covered with foil and stirred for a further 24 hours.

The contents of the beaker were sieved through a 500µm mesh and the solid material (if present) was rinsed in saline and examined for nematodes. The filtrate was discarded.

### 3.4.3 Prevalence and Intensity of Nematode Infection

The prevalence (percentage of infected specimens from the entire sample) and mean intensity (mean number of parasites in each infected specimen) of infection were calculated for whole fish according to weight class, total length and sex.

### 3.4.4 Identification techniques

Identification of the nematodes was primarily based on the morphology of the internal organs using light microscopy. If this could not be seen (because the specimen was not an anisakid, or because it had been damaged), the nematodes were labelled as 'unidentified'. A subsample of the main species were fixed for examination of the external features with a scanning electron microscope (SEM).

#### 3.4.4.1 Gross Morphology

The gross morphology can be used to differentiate between *A. simplex* and *P. decipiens*. *A. simplex* is typically smaller (9-36mm long), off-white in colour and characteristically coils like a 'watch-spring' (Fig. 14a) (Smith and Wooten, 1984b). *P. decipiens* by contrast, is generally larger (9-58mm long) and of variable colour (from off-white to dark brown-red). This species forms irregular coils (Fig. 14b) (Smith and Wooten, 1984a). Identifications made using this method must be confirmed using microscopy due to the variability in the appearance of the species, but are useful for making quick, preliminary identifications.



Figure 14a: *A. simplex* forming characteristic 'watch-spring' coils.



Figure 14b: *P. decipiens* coiled in cod flesh. Note the darker colour and irregular coils.

#### 3.4.4.2 Light Microscopy: Whole Specimens

Fixation and clearing of the samples were carried out based on advice from Dr. Isabel Coombs (University of Glasgow) and Eileen Harris (Natural History Museum). Nematodes were fixed in hot (70-80°C) 70% ethanol and left for approximately 24 hours. They were then transferred into clove oil and left to clear for at least 12 hours. Identification was carried out using a stereo light microscope (magnification 40-100x), and was primarily based on differences in the morphology of the anterior digestive tract (Fig. 15) as described by Smith and Wooten (1984a-c). The nematodes were then separated by species, rinsed in acid-ethanol (0.5ml 100% acetic acid: 100ml 100% ethanol) to remove the clearing agent and transferred into vials of 70% ethanol for long-term storage. A subsample of nematodes was sent to Eileen Harris (NHM, London) to confirm their identities.

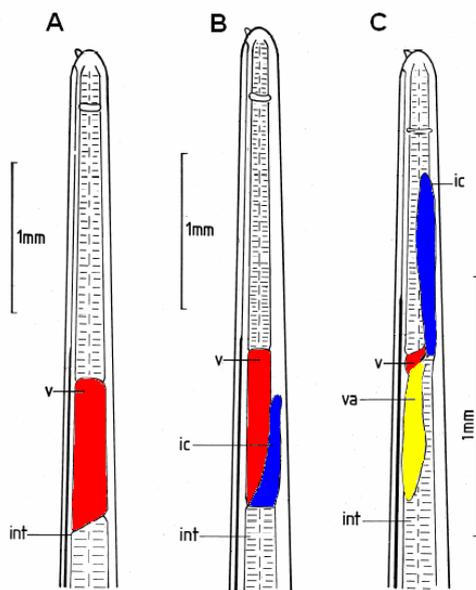


Figure 15: Differences in the morphology of the anterior digestive tract of the L3 stages of *Anisakis simplex* (A), *Pseudoterranova decipiens* (B) and *Hysterothylacium aduncum* (C).

Ventriculus (v) is shown in red, the intestinal caecum (ic) in blue and the ventricular appendix (va) in yellow. The intestine (int) is also indicated. Modified from Smith and Wooten (1984a-c).

#### 3.4.4.3 SEM

The external structure of a subsample of *A. simplex* and *P. decipiens* was examined with SEM. The identities were confirmed using Weerasooriya *et al.* (1986). These samples were fixed in 2% p-formaldehyde/ 2.5% gluteraldehyde with 0.1M PO<sub>4</sub> buffer (4% sucrose/1.5% NaCl) for approximately two hours before being transferred to 0.1M PO<sub>4</sub> (8% sucrose) buffer rinse overnight. The samples were stored at 0-4°C until needed.

The samples were rinsed three times in 0.1M PO<sub>4</sub> (8% sucrose) buffer for five minutes each to remove all traces of gluteraldehyde from the specimens. The buffer was then drained until it only just covered the samples, and 1% osmium tetroxide with 0.1M PO<sub>4</sub> (8% sucrose) buffer was added to the samples and left for one hour. After this time, the samples were washed three times in distilled water (10 minutes each) and progressively dehydrated through 30%, 50%, 70%, 90%, absolute (washed twice) and dried absolute acetone (CH<sub>3</sub>CHCO<sub>3</sub>). After dehydration, the samples were critical-point dried in liquid O<sub>2</sub> for 1 hour 20 minutes and mounted on aluminium stubs with double-sided copper tape and silver paint. They were coated in a gold and palladium mixture (30-40nm thick) using a Polaron SC515 SEM coating system (20mA, 1.5kV).

#### **3.4.5 Data Analysis**

The data were analysed using basic statistical tests in Minitab 15. The distribution trends were examined using Kruskal-Wallis analysis (with a paired comparisons *post hoc* test where appropriate) as the assumptions could not be met for ANOVA. Paired t-tests were used to compare the weights of the left and right fillets, and correlation analyses were performed to test the effects of fishing area and vessel and somatic condition. 95% confidence was considered significant in all analyses.

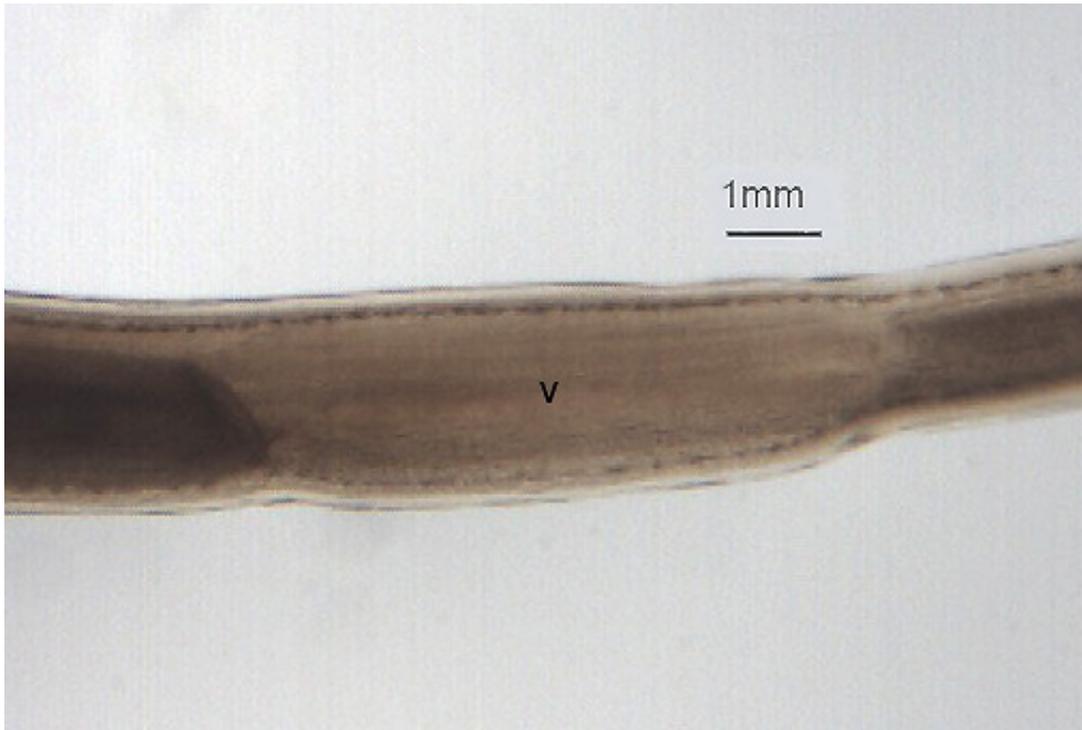
### 3.5 Results

#### 3.5.1 Identification of the parasites

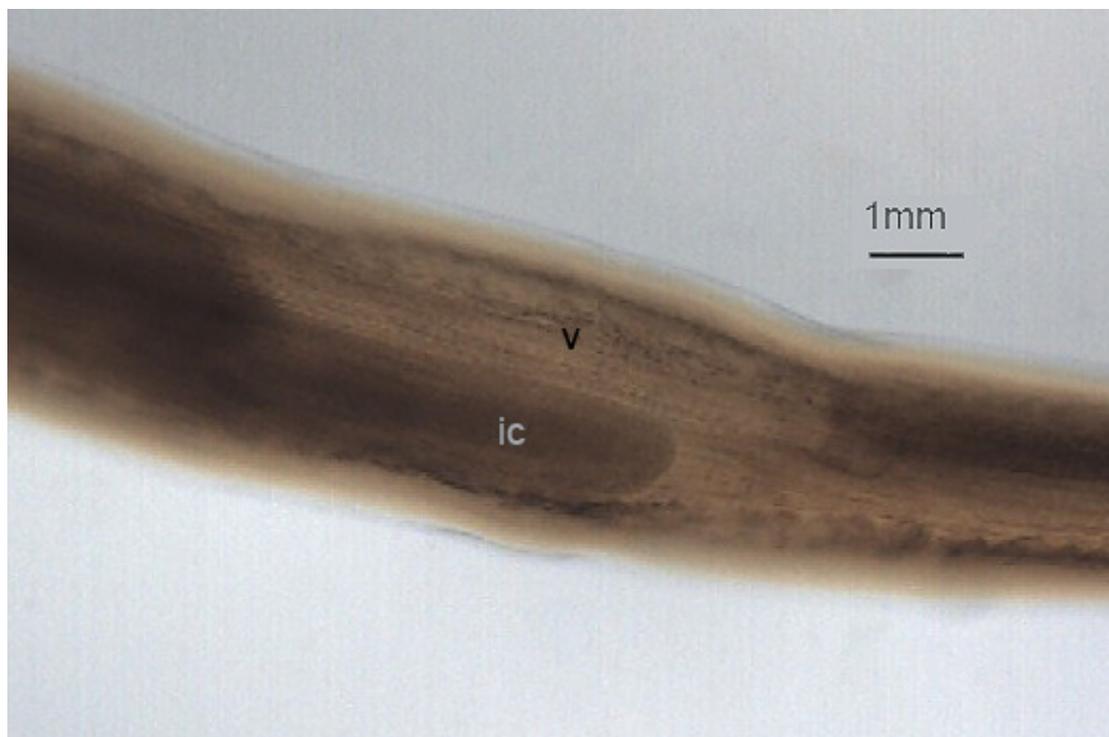
Three species of anisakid nematode were identified from a total of 4920 nematodes. These were the third stage larvae of *Pseudoterranova decipiens* and *Anisakis simplex* and both larval and adult stages of *Hysterothylacium aduncum*. Of these, only *P. decipiens* and *A. simplex* were found in the musculature. The anterior digestive tracts of *A. simplex*, *P. decipiens* and *H. aduncum* are shown in Figures 16-18 to highlight the differences in morphology.

A detailed comparison of the external structures of *A. simplex* and *P. decipiens* was made using SEM. These species are shown in Figure 19 (a-f), highlighting differences in the morphologies of the head structure (anterior), terminal mucron (posterior) and differences in the cuticular appearance (mid-cuticle). Comparisons to pictures in Weerasooriya *et al.* (1986) confirmed that the samples were *A. simplex* and *P. decipiens* (L3).

From the samples sent to NHM, all *A. simplex* specimens were confirmed as such, as were all *P. decipiens* except one. The correct identities of the nematodes in this subsample are shown in Table 3. The specimen of *A. simplex* that was incorrectly identified as *P. decipiens* is marked with an asterisk.

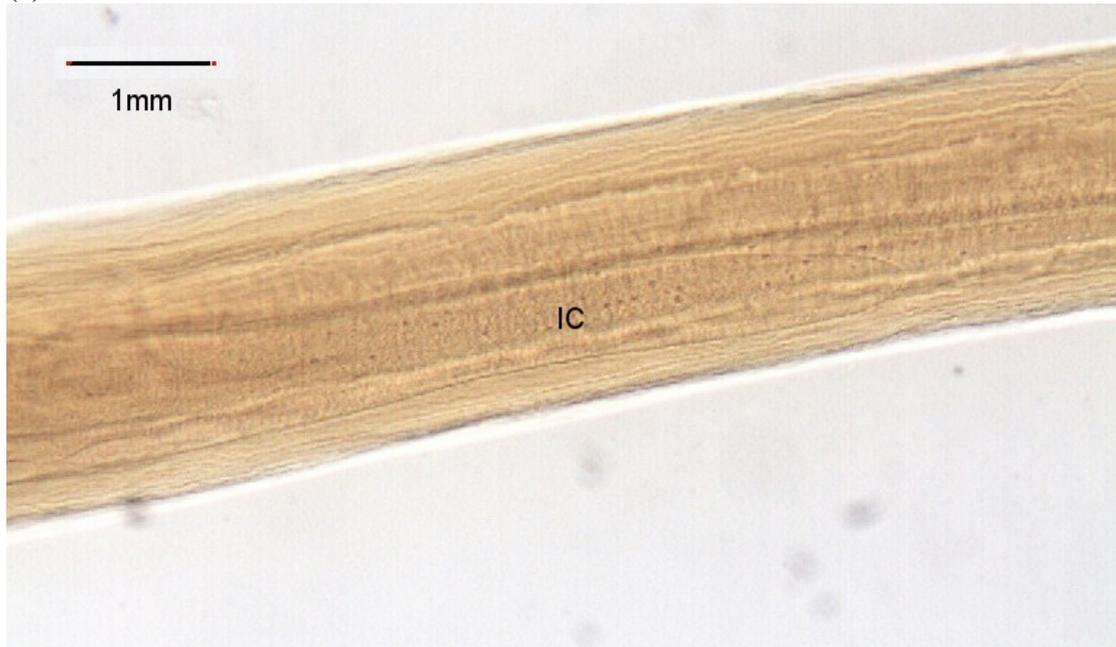


*Figure 16:* Ventriculus (v) of *A. simplex*. Notice there is no ventricular appendix or intestinal caecum.

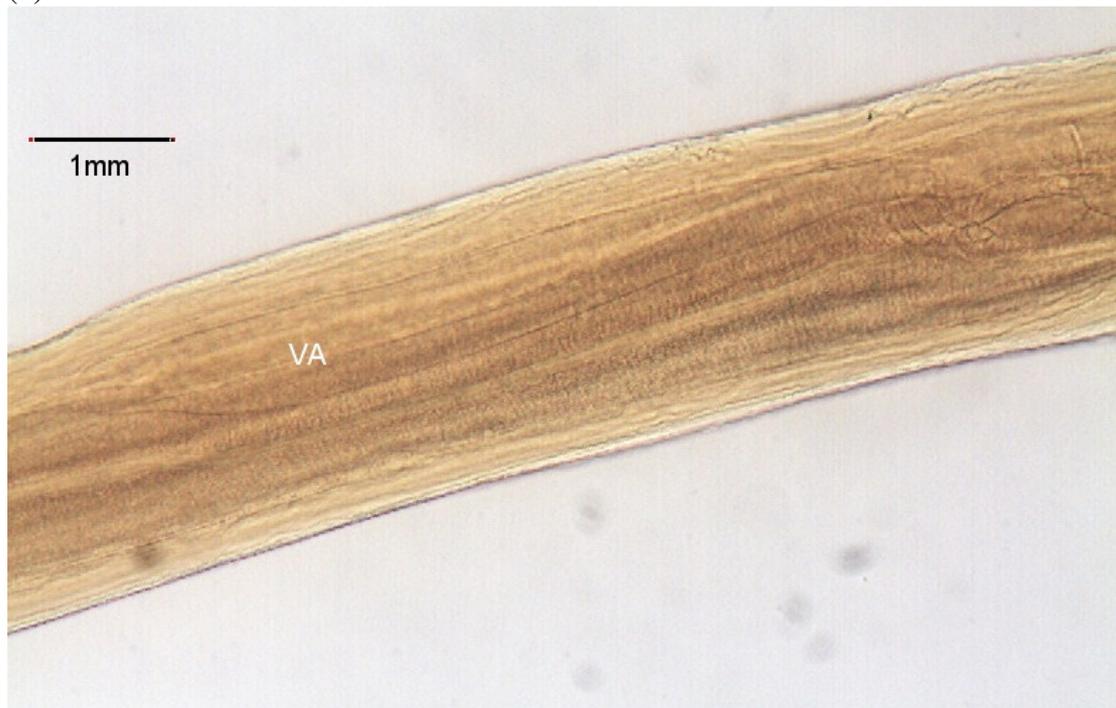


*Figure 17:* Ventriculus (v) of *P. decipiens*. The intestinal caecum (ic) is indicated.

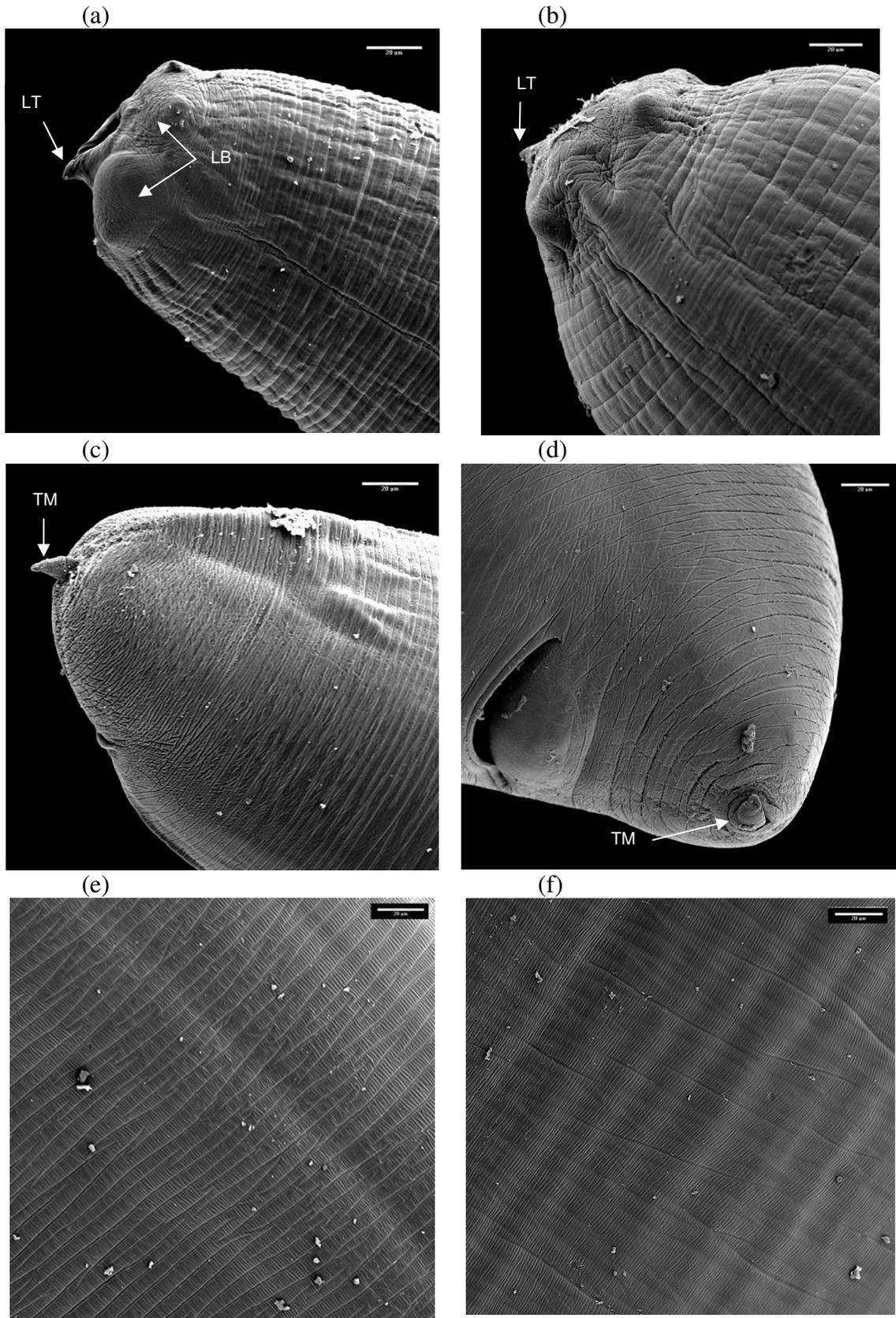
(a)



(b)



*Figure 18: (a) Intestinal caecum (ic) and (b) ventricular appendix (va) of *H. aduncum* (specimen from NHM).*



*Figure 19* (a)-(f): Scanning electron micrographs. Scale bar = 20μm. LT = larval tooth; LB = lip bulge; TM = terminal mucron.

Figure 19 (a) Anterior end of *A. simplex*. The larval tooth is visible, as is the dorsal lip bulge, and one of the two subventral lip bulges.

Figure 19 (b) Anterior end of *P. decipiens*. Structure is similar to *A. simplex*.

Figure 19 (c) Posterior end of *A. simplex* showing the terminal mucron.

Figure 19 (d) Posterior end of *P. decipiens* showing the terminal mucron.

Figure 19 (e) Cuticle of *A. simplex* with irregular transverse grooves and parallel longitudinal grooves.

Figure 19 (f) Cuticle of *P. decipiens*. Note the two forms of transverse groove.

*Table 3:* Corrected identities of the nematodes sent to the Natural History Museum compared to my initial identification. \*Indicates the single *A. simplex* specimen that was incorrectly identified as *P. decipiens*.

<b>ID No.</b>	<b>Initial Identification</b>	<b>NHM Identification</b>	<b>No.</b>
TROS 2	<i>Anisakis simplex</i>	<i>Anisakis simplex</i>	21
ICE4 COD2-LM	<i>Anisakis simplex</i>	<i>Anisakis simplex</i>	8
ICE4 COD2-LM	<i>Pseudoterranova decipiens</i>	<i>Pseudoterranova decipiens</i>	13
ICE4 COD2-CM	<i>Pseudoterranova decipiens</i>	<i>Pseudoterranova decipiens</i> and <i>Anisakis simplex</i> *	5
ICE2 COD1-V	<i>Pseudoterranova decipiens</i>	<i>Pseudoterranova decipiens</i>	5
ICE7 COD1-V	<i>Contracaecum osculatum</i>	<i>Hysterothylacium aduncum</i> and <i>Cucullanus cirratus</i>	2
ICE5 COD3-V	<i>Contracaecum osculatum</i>	<i>Hysterothylacium aduncum</i> or <i>C. osculatum</i>	2
ICE5 COD3-V	<i>Contracaecum osculatum</i>	<i>Hysterothylacium aduncum</i> or <i>C. osculatum</i>	1
ICE5 COD3	<i>Contracaecum osculatum</i>	<i>Hysterothylacium aduncum</i> or <i>C. osculatum</i>	2
ICE7 COD1-V	Unknown	<i>Hysterothylacium aduncum</i>	8
ICE5 COD1-V	Unknown	<i>Hysterothylacium aduncum</i> and <i>Echinorhynchus gadi</i>	15
ICE5 COD2	Unknown	<i>Hysterothylacium aduncum</i> (L4)	2

*A. simplex*, *P. decipiens* and *H. aduncum* accounted for 72.8% of all nematodes found (Fig. 20). The remainder ('unknown') were unidentifiable to species level.

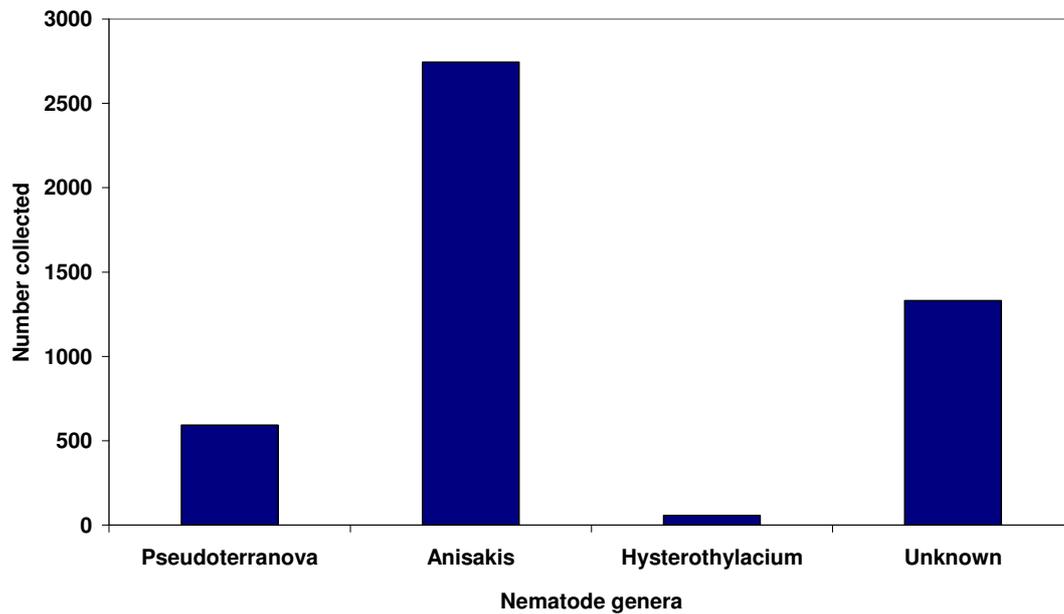


Figure 20: Total number of nematode larvae collected from each genus.

### 3.5.2 Sampling Efficiency

The numbers and percentage of nematodes detected in a sub-sample of 6 sides of cod using each detection method is shown in Table 4. The pepsin-HCl digestion was assumed to reveal 100% of all nematodes present.

Table 4: Sampling efficiency of three detection techniques from each muscle region.

		<b>Candling</b>	<b>Candling &amp; Slicing</b>	<b>Digestion</b>
<b>No. nematodes</b>	<b>Loin</b>	3	0	0
	<b>Tail</b>	3	0	0
	<b>Abdominal flap</b>	26	4	3
<b>% detected</b>		78.1	12.5	9.4
<b>Cumulative %</b>		78.1	90.6	100

All nematodes from the 'loin' and 'tail' regions were *P. decipiens*, and it appeared that candling alone is able to detect 100% of this species. With such a small sample

size however, this should be viewed with caution. The remaining nematodes were *A. simplex*, and approximately 91% were detected using a combination of candling and slicing.

### 3.5.3 Infection Levels

The prevalence and mean intensity of infection of *A. simplex* and *P. decipiens* in the entire body (muscle and viscera) of whole fish (n = 22) is shown in Table 5. Table 6 shows the prevalence and mean intensity in the fillets of the same fish. The prevalence and mean intensity of nematode infections in whole fish by fillet weight is shown in Table 7, and the number of nematodes per kilogram of fillet is shown in Table 8. Table 8 shows values for both the overall nematode burden and for infected fillets only.

Table 5: Prevalence and mean intensity of nematode infection in whole cod (muscle and viscera).

		Number of cod	Prevalence		Mean Intensity	
			<i>Anisakis</i>	<i>Pseudoterranova</i>	<i>Anisakis</i>	<i>Pseudoterranova</i>
<b>Sex</b>	M	9	100	100	249.44	21.11
	F	11	100	72.7	31.45	23.00
<b>Length (cm)</b>	50-69	15	100	80	16.73	11.27
	70-89	6	100	100	390.50	37.50
<b>Weight (g)</b>	1001-2000	9	100	66.7	21.67	8.67
	2001-3000	6	100	100	9.33	19.50
	>3001	6	100	100	390.50	37.50

Table 6: Prevalence and mean intensity of nematode infection in the fillets of whole cod.

		Number of cod	Prevalence		Mean Intensity	
			<i>Anisakis</i>	<i>Pseudoterranova</i>	<i>Anisakis</i>	<i>Pseudoterranova</i>
<b>Sex</b>	M	9	0	33.3	0	3.67
	F	11	18	36.4	2.00	8.00
<b>Length (cm)</b>	50-69	15	13.3	33.3	2.00	7.60
	70-89	6	0	33.3	0	2.50
<b>Weight (g)</b>	1001-2000	9	0	22.2	0	7
	2001-3000	6	33.3	50	2.00	4.17
	>3001	6	0	33.3	0	2.50

Table 7: Prevalence and mean intensity of nematode (both species) infection by fillet weight of whole cod.

<b>Fillet weight</b>	<b>No. fillets</b>	<b>Prevalence</b>	<b>Mean intensity</b>
101-200g	15	33.333	3.60
201-300g	14	69.231	4.00
301-400g	6	33.333	1.50
400+	7	14.286	3.00

Table 8: Number of nematodes (both species) per kg of fillet (whole cod).

<b>Fillet weight</b>	<b>No. fillets</b>	<b>Nematodes/kg fillet</b>	<b>Nematodes/kg infected fillet</b>
101-200g	15	7.66	22.98
201-300g	14	9.48	12.63
301-400g	6	0.00	0.00
400+	7	1.23	3.07

The mean intensity of infection in the muscle and viscera with *P. decipiens* appeared to increase with weight and length class. This trend appeared to reverse when only the fillets were examined.

A similar trend was apparent for *A. simplex* in the viscera and muscle, and the mean intensity of infection appeared to increase with length class. There were no clear trends for weight class.

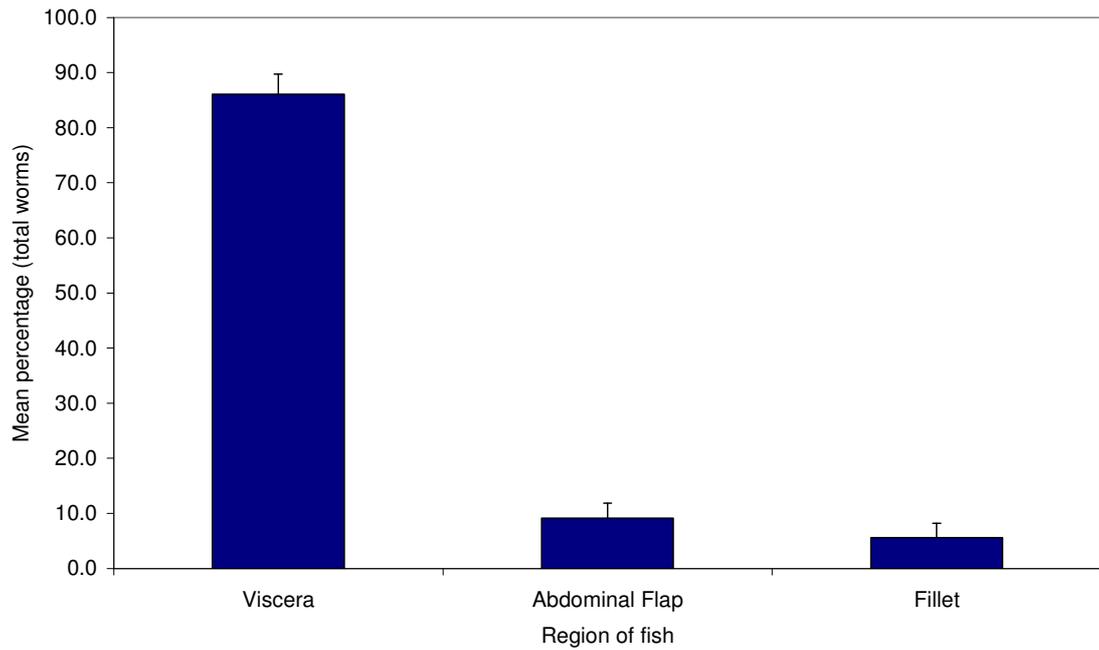
Females had a higher prevalence and mean intensity of both parasite species when only considering the fillet portion of the muscle.

There was no clear trend in the prevalence or mean intensity of infection based on fillet weight, but it appeared that there was a reduction in the number of nematodes per kg fillet in fillets of 300g or more, compared to those between 100g and 300g.

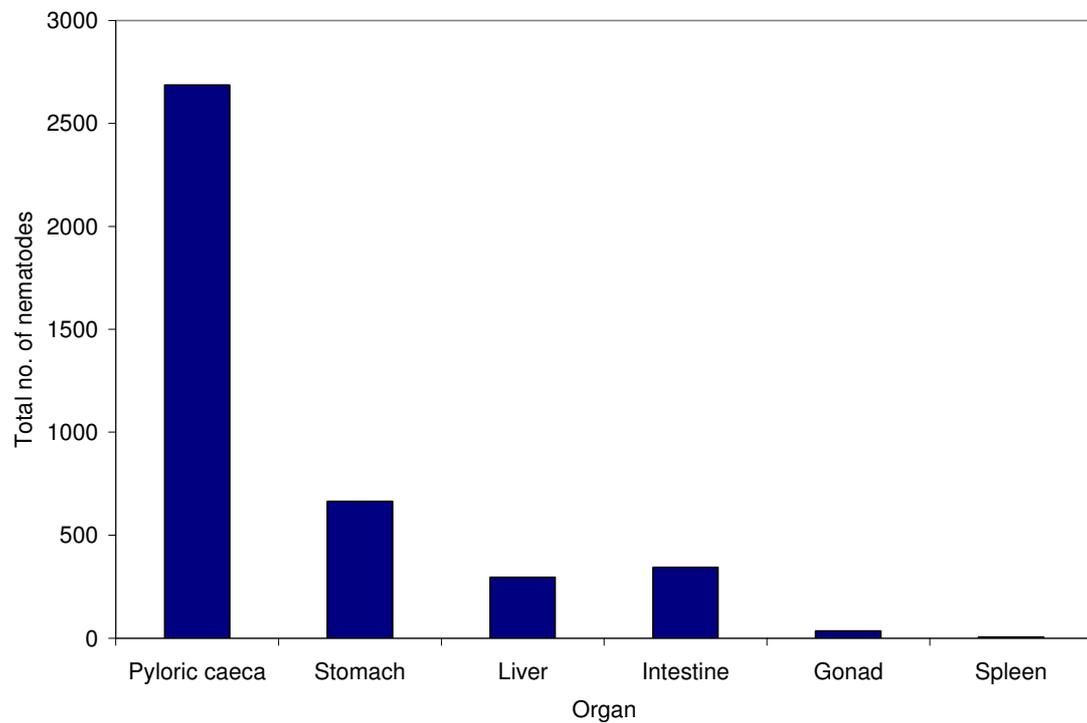
### 3.5.4 Distribution

Significantly more nematodes were found in the viscera of the 21 whole fish (mean = 86.1%) than in either the abdominal flaps (mean = 9.1%) or fillets (mean = 5.6%) ( $p = 0.000$ ) as shown in Figure 21. There was no significant difference between the numbers of nematodes found in the abdominal flaps and fillets of whole fish ( $p = 0.0658$ ).

Within the viscera, two-thirds of the nematode larvae were recovered from the pyloric caeca (66.6%), 16.5% from the stomach, 8.5% from the intestine and 7.34% from the liver. The total numbers of nematodes in each visceral organ are shown in Figure 22. *Post hoc* analysis showed no significant difference between the nematode burdens in the pyloric caeca and liver, liver and stomach, stomach and intestine, or between the intestine, spleen and gonads. All other comparisons were significant.



*Figure 21:* The mean percentage of nematodes found in the main regions of whole cod ( $n = 22$ ).  $H = 43.95$ ,  $df = 2$ ,  $p = 0.000$ . Error bars show 1 standard error.



*Figure 22:* Total numbers of nematodes recovered from each visceral organ of all whole cod ( $n = 22$ ).

When all cod were compared (whole and gutted), significantly more nematodes were found in the abdominal flaps than the fillets (mean = 3.5 and 1.01 respectively) ( $p = 0.000$ ) as shown in Figure 23. There was no significant difference between the right and left sides of the cod. The fillets taken from the right side of the cod were significantly heavier ( $p = 0.004$ ), but it is unclear whether this would have affected the nematode numbers as the mean difference between the sides was only 20g.

The precise distribution within the musculature varied depending on the species of nematode, as shown in Figure 24. There were significant differences between the numbers of *P. decipiens*, *A. simplex* and 'unknown' nematodes in the fillets and abdominal flaps ( $p = 0.000$ ). *Post-hoc* analysis showed that there were significantly more *A. simplex* in the abdominal flaps than in the fillet ( $p = 0.000$ ), and more *A. simplex* in the abdominal flaps than both *P. decipiens* ( $p = 0.000$ ) and unknown nematodes ( $p = 0.000$ ). There were no significant differences between species in the fillets, or between numbers of *P. decipiens* or unknown nematodes in the fillets and abdominal flaps.

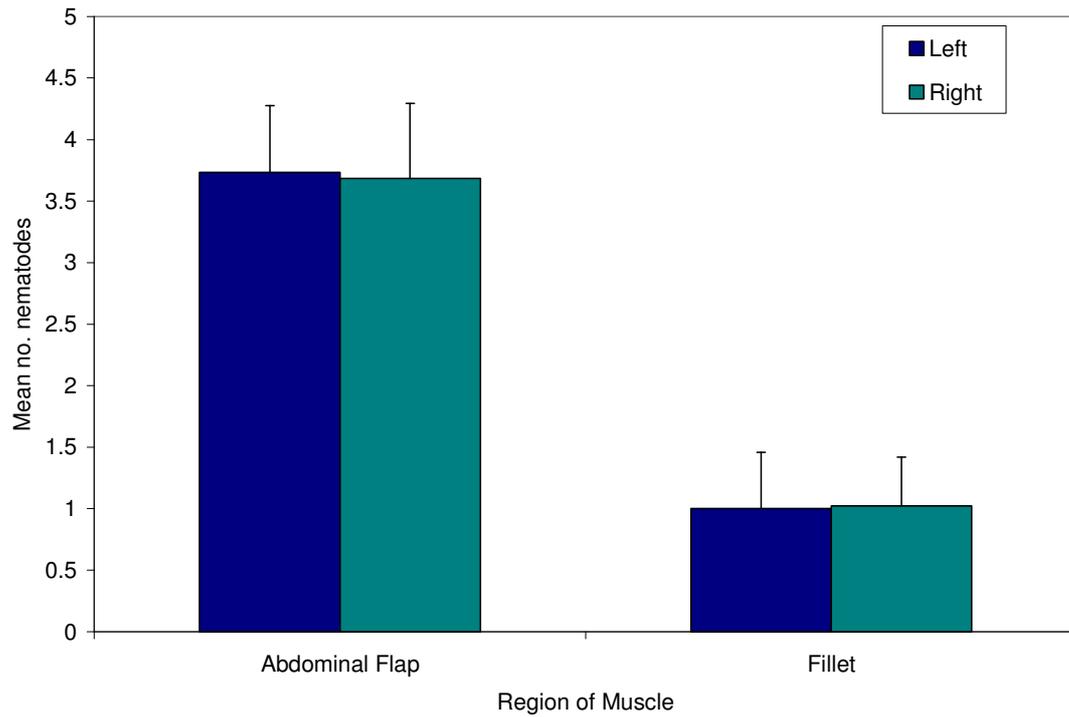


Figure 23: Mean numbers of nematodes in each part of the musculature and from each side of the cod (n = 46).  $H = 46.84$ ,  $df = 1$ ,  $p = 0.000$ . Error bars show 1 standard error.

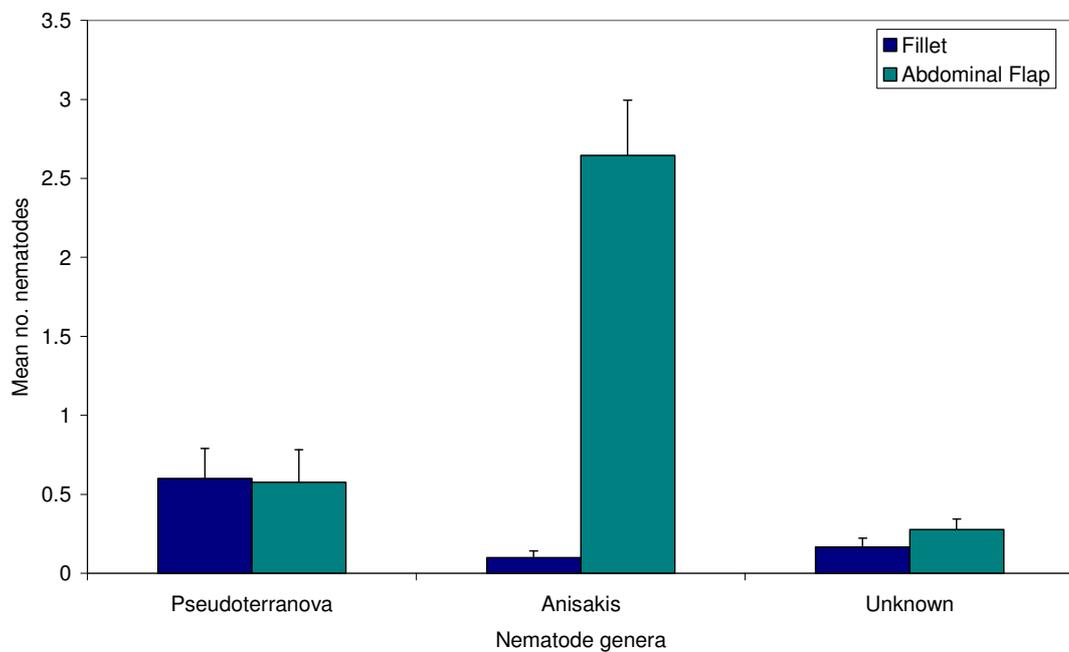


Figure 24: Numbers of each nematode genus in the abdominal flaps and fillets of cod (n = 46).  $H = 159.71$ ,  $df = 5$ ,  $p = 0.000$ . Error bars show 1 standard error.

### **3.5.5 Other Trends**

#### ***3.5.5.1 Somatic condition***

The somatic condition index for Atlantic cod is a means to compare the overall condition of individual cod, and can be expressed by:  $(\text{weight} / \text{length}^3 \times 100)$ . The higher the number, the better the condition of the animal.

There was no significant difference between the somatic condition factor and the month of capture ( $p = 0.093$ ) (Fig. 25).

There was no correlation between somatic condition factor and either the total number of nematodes in the body ( $R = -0.042$ ,  $p = 0.856$ ) or the number of nematodes in the fillets ( $R = -0.008$ ,  $p = 0.974$ ) (Figs. 26 and 27).

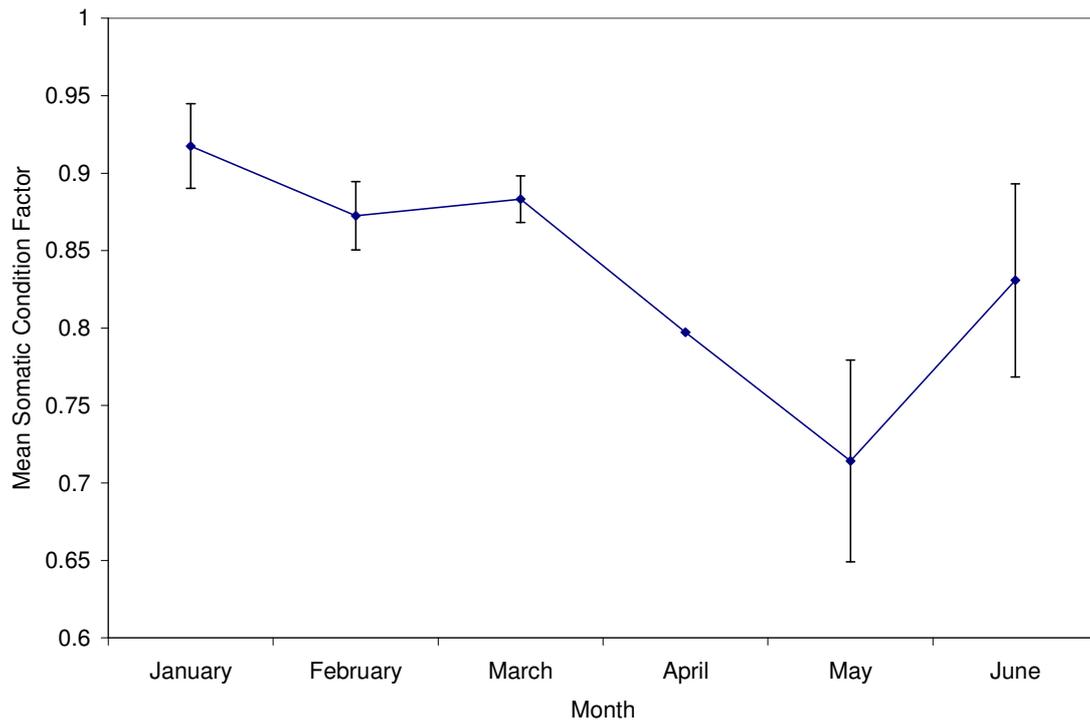
#### ***3.5.5.2 Body Regions***

There was no significant correlation between the numbers of nematodes on the viscera and the entire muscle ( $p = 0.577$ ) or the fillets ( $p = 0.388$ ).

#### ***3.5.5.3 Vessels and Capture Areas***

There was no significant difference between the numbers of nematodes in cod muscle (untrimmed fillets) and the type of vessel that caught them (day boats or factory boats;  $p = 0.489$ ). There was no difference between the numbers of nematodes in the (trimmed) fillets and type of vessel ( $p = 0.465$ ).

The capture area had no significant effect on the number of nematodes in untrimmed or trimmed fillets ( $p = 0.849$  and  $0.157$  respectively). There was no effect on the total nematode burden in whole (ungutted) cod either ( $p = 0.308$ ).



*Figure 25:* Mean somatic condition factor during each month of capture (whole cod only;  $n = 22$ ).  $H = 9.44$ ,  $df = 5$ ,  $p = 0.093$ . Error bars show  $\pm 1$  standard error.

#### ***3.5.5.4 Stomach contents***

Of the 22 whole cod examined, 17 had food in their stomachs. Two of these (ICE8 COD2 and ICE10 COD2) only contained the bait from a hook (piece of herring), while the remainder contained a variety of organisms, the majority of which were benthic macro- and megafauna. Two fish had what appeared to be squid mantles in their stomachs, but the rest of the animal had been digested so this could not be confirmed. These have not been formally identified or quantified, but brief descriptions of the stomach contents are given in Table 9.

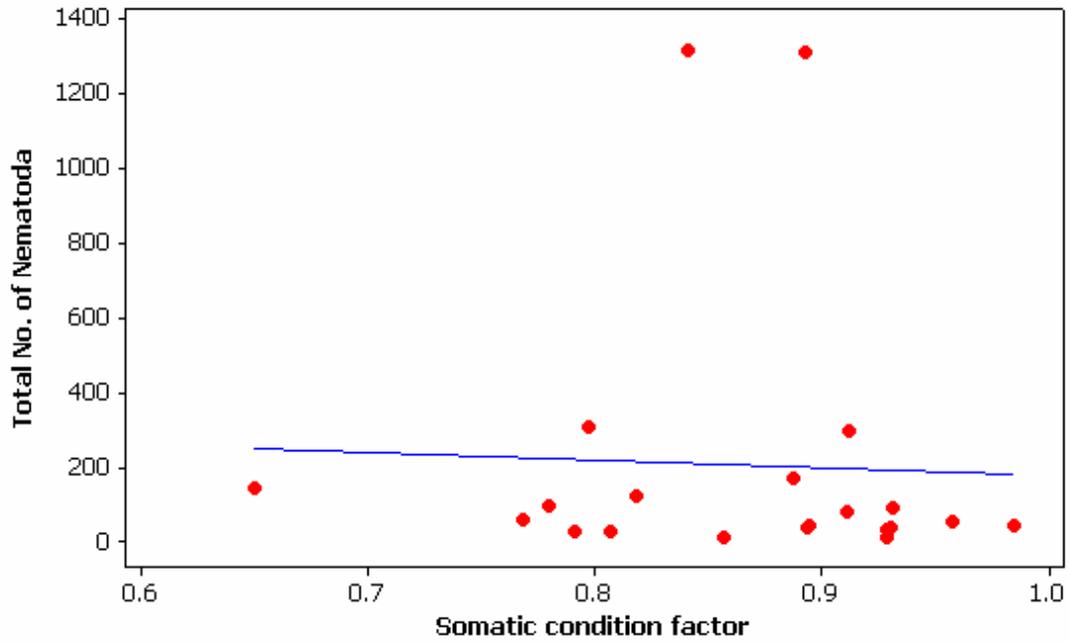


Figure 26: The total no. of nematodes correlated against the somatic condition factor of whole cod (n = 22).  $R = -0.042$ ,  $p = 0.856$ .

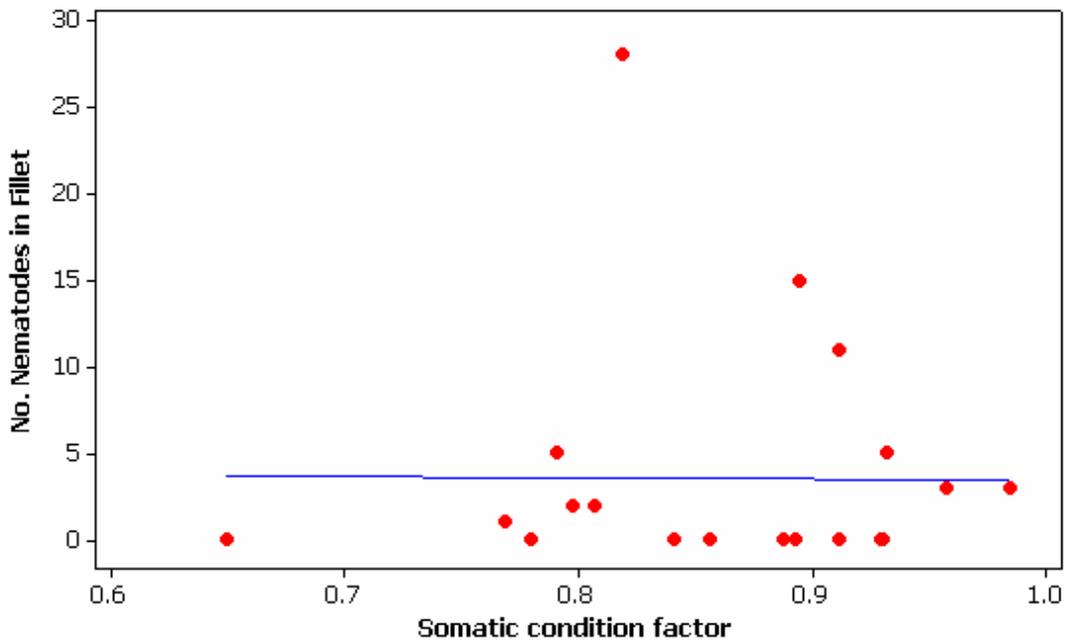


Figure 27: The no. of nematodes in the fillets correlated against the somatic condition factor of whole cod (n = 22).  $R = -0.008$ ,  $p = 0.974$ .

Table 9: Short descriptions of the stomach contents of each whole cod.

Sample ID	Stomach Contents
ICE1 COD1	Prawns
ICE1 COD2	Prawns, bait
ICE1 COD3	Brittle stars, various benthic macrofauna
ICE1 COD4	Prawns, squat lobster
ICE2 COD1	Prawns
ICE2 COD2	Possibly squid mantle
ICE4 COD1	Squat lobster
ICE4 COD2	Brittle stars, squat lobster claw
ICE5 COD1	Squat lobster, prawns, bivalve shell, barnacle shell
ICE5 COD2	Squat lobster claw
ICE5 COD3	Prawns, squat lobster
ICE7 COD1	Fragments (e.g. brittle star legs)
ICE8 COD2	Bait, nematodes (poss. <i>H. aduncum</i> )
ICE10 COD2	Bait
ICE12 COD1	Possibly squid mantle and one shrimp. Badly digested.
ICE16 COD1	Various crustaceans including squat lobsters and prawns
ICE16 COD2	Various crustaceans including hermit crab claws, squats, prawns

## 3.6 Discussion

### 3.6.1 Identification

The results of this study confirm that the species of nematode present in the musculature of the cod were the third larval stages of *Pseudoterranova decipiens* and *Anisakis simplex*. Both species can be pathogenic to humans if eaten live, and it is therefore important that they are removed or destroyed before the cod is sold to consumers.

*Hysterothylacium aduncum* was present, but only on the cod viscera. Some adult *H. aduncum* were also found by chance within the intestine of some cod but these were not included in the final counts as the internal surfaces of the viscera were not examined formally.

Based on the identities provided by the Natural History Museum, it appears the identifications of *P. decipiens* and *A. simplex* were mostly accurate. One specimen of *A. simplex* was misidentified as *P. decipiens*, but given the clear morphological differences between the two species it is likely that this was a simple error, and is unlikely to significantly affect the final numbers.

Two additional species were identified at the NHM: a nematode, *Cucullanus cirratus* (Müller, 1777) and an acanthocephalan, *Echinorhynchus gadi* Zoega in Müller, 1776. *C. cirratus* has been previously reported from the intestine (Hemmingsen and MacKenzie, 2001) and pyloric caeca (Kjøie, 2000) of Atlantic cod, and is an internal parasite of fish. *E. gadi* is also an extremely common parasite of Atlantic cod, in which it is believed to be relatively benign (Wayland *et al.*, 2005). It is an internal parasite, which may or may not mature within the intestine. There is no evidence that either of these species is pathogenic to humans or has been found in the musculature.

As previously discussed, *A. simplex* and *P. decipiens* can be readily distinguished by examining the different morphologies of the intestine and ventriculus. However, molecular techniques have recently shown that these are actually species complexes containing a number of cryptic species (e.g. Mattiucci *et al.*, 2007).

The *A. simplex* species complex currently contains two species: A (named *A. pegreffi*) and B (*A. simplex sensu stricto*) of which *A. simplex s. str.* is most common in the NE Atlantic (Nascetti et al, 1986). These are ‘true’ species, in that mating barriers exist between them and no fertile F1 offspring were produced in crosses. Additionally, *A. pegreffi* is mostly found in pelagic hosts, whereas *A. simplex s. str.* is mainly present in benthic or demersal species (Mattiucci et al, 1997). Based on its ecology, we may expect that *A. simplex s.str.* is present in these cod rather than *A. pegreffi*, but this would need to be confirmed by molecular analysis or culture of larvae to adult stage.

The *P. decipiens* species complex contains five cryptic species (A-E), of which A-C are found in the north Atlantic (McClelland, 2002). Species A and B are found around northern Europe and Iceland; C is only found in the Barents Sea. Species A (may be renamed *P. krabbei*), is most frequently found in grey seals (*Halichoerus grypus*), whereas species B (may be renamed *P. decipiens s. str.*) is most frequently found in common seals (*Phoca vitulina*), and rarely in grey seals (Apsholm et al., 1995). This may represent a competitive interaction as either species of nematode may be found in either seal where the other parasite is not present (Paggi et al., 2000). Both *H. grypus* and *Phoca vitulina* are found around Icelandic coasts, which makes distinguishing between *P. decipiens* A and B impossible without molecular data.

Since the identifications in the present study were made using microscopy rather than molecular techniques, it was impossible to distinguish between cryptic species within the complexes. It is possible that these cryptic species may have different host preferences, environmental tolerances or pathogenicities and further study into these potential differences would be useful.

Additionally, the use of molecular techniques may be beneficial to the fishing and processing industry as a forensic tool for identifying nematodes that are returned as customer complaints. Since these nematodes are often in poor condition, traditional identification techniques are not always effective (Eileen Harris, *pers. comm.*), and molecular sequencing may be a suitable alternative.

### 3.6.2 Prevalence and Mean Intensity of Infection

Both *A. simplex* and *P. decipiens* were less prevalent within the cod fillets (0-50%) than in the 'viscera and musculature' (66.7-100%; where 'musculature' included both fillets and abdominal flaps). This is perhaps not surprising as the fillets are a subset of the 'viscera and muscle', but indicates that not all infected cod will have nematodes in the flesh. The mean intensity of infection was also lower in the fillets than in the combined viscera and muscle.

There was no clear trend in prevalence or mean intensity of infection with fillet weight, but there did appear to be an effect on the mean number of nematodes per kg fillet. The number per kg fillet is effectively a combination of prevalence and mean intensity data and is commonly used by the processing industry to assess the quality of their products (Linda Wood, *pers. comm.*). In particular, there appeared to be a reduction in the mean number of nematodes in fillets over 300g when compared to those under 300g. This could prove useful in sourcing fish with a naturally lower parasite burden.

The two largest cod specimens had a particularly high intensity of *A. simplex* in the viscera (mean = 1012 nematodes). This may have skewed the data in this respect, and the values should be treated cautiously. However, there were no nematodes in the fillets of these fish, suggesting that other factors are involved in the migration of nematodes to the musculature.

The prevalence, mean intensity and mean length of *A. simplex* has been shown to increase with host age in cod (Hemmingsen *et al.*, 2000, Strømnes and Andersen, 2003). Platt (1975) studied *A. simplex* and *P. decipiens* in cod from Icelandic waters and found that host length, age and gutted weight were significant predictors of the parasite burden in the muscle of the cod, although the relationship was complex and non-linear. A survey in Scottish waters by Wootten and Waddell (1977) also showed a similar trend of increasing infection with age and length of cod. Des Clers (1991) described a model based on the Firth of Clyde cod fishery which showed a relationship between host (cod) age and length and parasite burden as a functional relationship between the size of the fish and the amount of food consumed. Larger

fish ate more, and were therefore more susceptible to ingesting parasite larvae and becoming infected.

Such trends may be explained by the long lifespan of the parasite and increased infection rates with the onset of piscivory. As cod grow, they switch from a benthivorous to piscivorous diet, which makes them more likely to ingest high numbers of anisakid nematodes (through infected primary fish hosts), resulting in high prevalence and intensity in the viscera. However, it has also been suggested that the impenetrability of the stomach wall, coupled with a greater physical distance between the viscera and musculature in larger fish mean that fewer nematodes are able to penetrate the muscle tissue (Smith, 1984, McClelland, 2002), hence the numbers in the flesh decrease with size. Additionally, large cod may simply be too big to be eaten by the definitive host, meaning parasites will accumulate over its lifetime (McClelland, 2002). This is purely conjecture however, and further study would be required to confirm such a hypothesis.

Further work in this area could be carried out quite easily by commercial processors, and could quickly provide additional data to confirm the trends seen in this study.

### **3.6.3 Distribution**

The distribution of anisakids within the cod viscera and musculature was similar to that described in previous studies. The majority of all parasites were found on the viscera, particularly the pyloric caeca and liver. Other authors have shown similar trends, although more larvae have typically been reported from the liver than the pyloric caeca. Bratney and Bishop (1992) for example, reported that the majority of *A. simplex* from pepsin-HCl digested cod were found in the liver (31.7 - 47.1%) or around the other viscera rather than the muscle.

*A. simplex* has mostly been found in the abdominal flaps and viscera of cod (e.g. Platt, 1975, Bratney and Bishop, 1992, Strømnes and Andersen, 2000) which supports the findings of this study. Hauksson (1989) reports higher numbers of 'roundworm' from the nape of the cod, but does not state which species are present.

By contrast, *P. decipiens* has a more homogeneous distribution. Previous work suggests that *P. decipiens* is relatively rare in the viscera, and is more common in the fillet portion of the musculature than the abdominal flaps (Platt, 1975, Bratney and Bishop, 1992). There is no evidence from this study that *P. decipiens* is more abundant in either the fillet or abdominal flap, but this may be due to the smaller sample size used in this study. Platt (1975) also reports that most of his identifications were made by eye rather than examining the internal morphology under a microscope, which could have allowed small *P. decipiens* to be overlooked due to their similarity to *A. simplex* which may have skewed his data. It is unclear whether the specimens collected by Bratney and Bishop (1992) were all identified under a microscope or mostly by eye.

There was no evidence that more nematodes were present in one side of the cod than the other, although previous authors have reported higher numbers occurring in the left side of cod (e.g. Bratney and Bishop, 1992, Smith and Hemmingsen, 2003). Smith and Hemmingsen (2003) found that 54.8% and 55.5% of all the ascaridoid nematodes in two sample groups were present in the left-side musculature. These authors hypothesised that this distribution was related to the presence of the liver in the left side of the body, either because the liver is the first organ encountered by the nematodes after penetrating the stomach wall, or because the larvae had a natural preference for the liver tissue as a microhabitat. However, even if there is a difference between the numbers of parasites in each side of the body, the difference is probably too slight to provide a commercially useful solution to the problem.

#### **3.6.4 Other Trends**

There was no difference in the numbers of nematodes found in the flesh of cod caught by factory boats or dayboats. The different types of boats process their catches differently; in particular, cod caught by dayboats is not gutted for several hours, whereas cod from factory boats are gutted within approximately 30-60 minutes of capture. If nematode larvae migrate from the viscera to the flesh post-capture, these different practices could have affected the number of nematodes found in the muscle. Since there is no difference between the types of boat, it is reasonable to conclude that post-capture migration from the viscera does not occur within this timescale. Whether

migrations could occur within the first 60 minutes of capture or require a longer time than was available would require further study of the Icelandic fishery.

Smith and Wootten (1975) and Smith (1984) recorded significant post-capture migration of *A. simplex* from the viscera to the muscle of herring and mackerel. Such behaviour has never been demonstrated in 'lean' fish (e.g. gadoids) and it has been suggested that this could be related to the different distribution of lipids in the fish. Gadoids store most of their lipid in the liver (e.g. Falch *et al.*, 2006), whereas 'fatty' fish like herring and mackerel store much of their lipid in the muscle. If excapsulation of the nematodes (which must occur prior to migration) is influenced by the presence of lipid (Smith, 1984), the nematodes in cod are unlikely to move towards the flesh, and may be expected to remain in the viscera. Nonetheless, the stimulus causing migration to the muscle is still unknown, and would require additional investigation.

There was no difference in the number of nematodes in cod caught from the different sea areas. Guðmundsson *et al.* (2006) plotted the mean number of nematodes in trawled cod between 2001 and 2005 in four three-month periods. They found that the overall distribution of nematodes in cod varied considerably between periods, but would remain at relatively constant levels in the regions examined in this study throughout the year (approx. 6-9 nematodes/ kg fillet). These differences are likely related to the movements of the cod, rather than any changes in the nematode burden within individual fish. *A. simplex* can live for several years within its fish host (Smith, 1984, Hemmingsen *et al.*, 1993), as can *P. decipiens* (McClelland, 2002), and seasonal effects at this level are therefore unlikely.

The somatic condition of cod has been shown to vary seasonally in the Gulf of St. Lawrence, with the highest values generally occurring in October-November and lowest in April during the spawning season (Mello and Rose, 2005). No such trends were recorded in the present study although they may have been expected, particularly after the spawning season in April. This could simply be a result of high variation between samples and the low sample sizes for each month of the study. A larger long-term investigation would be required to determine whether there is a genuine seasonal difference in condition.

The stomach contents largely comprised benthic organisms, such as prawns, squat lobsters and brittle stars, which suggests that the majority of the cod captured were benthivorous. Atlantic cod switch from a benthivorous to piscivorous lifestyle as they age, and it has been hypothesised that piscivorous fish may accumulate more parasites as their prey contains higher numbers of nematodes (McClelland, 2002). This could not be confirmed by the results of the present study.

### 3.7 Chapter 3 Summary

A total of 4920 nematodes were examined from 21 whole cod, 24 gutted cod and two samples of nematodes removed by Tros. Three species of anisakid nematode were identified from this sample using light microscopy (whole mounts) and scanning electron microscopy (SEM): *Anisakis simplex* (larval stage), *Pseudoterranova decipiens* (larval stage) and *Hysterothylacium aduncum* (larval and adult stages). The identities were confirmed by an expert at the Natural History Museum, London. Of these species, only *A. simplex* and *P. decipiens* were recovered from the musculature. Both *A. simplex* and *P. decipiens* are known to be pathogenic to humans while alive, and have the potential to trigger allergic reactions in sensitized people.

In the whole cod, 85% of nematodes on average were recovered from the viscera (particularly from the pyloric caeca and liver).

Within the muscle, *A. simplex* tended to cluster in the abdominal flaps, whereas *P. decipiens* appeared to have a more homogeneous distribution. At present the abdominal flaps are removed from the cod fillets during processing at Tros, and it is likely that this is beneficial in reducing numbers of *A. simplex* in the final product.

Neither species of anisakid was more prevalent on one side of the cod compared to the other. Previous studies have recorded higher numbers in the left-side musculature of cod than the right, however even if they are correct, the differences are likely too small to be of commercial use.

The type of capture vessel (day or factory vessel), capture area, month of capture and the somatic condition of the cod had no effect on the numbers of nematodes present in the flesh. These results were based on a small data set, and should be viewed with caution. Additional work on the Icelandic fishery is necessary to confirm these trends.

Further study into the infection rates of cod could be carried out easily by MFG at their factory, and could quickly provide additional data on the trends in this study. Development of molecular identification techniques could be beneficial in determining the exact species of nematode that exist in the Icelandic cod population, and also the identities of nematodes returned to MFG as customer complaints.

## **Chapter 4: Behaviour of parasitic nematodes under varying experimental conditions**

### **4.1 Introduction**

The behaviour of parasitic nematodes has not been widely studied, and most investigations on larval *Anisakis simplex* and *Pseudoterranova decipiens* have focussed on their pathology and life cycles. Behavioural information is often extremely valuable however when attempting to manipulate or kill the organism in question.

One study into the behaviour of *P. decipiens* was carried out by Ronald (1960, 1962, 1963) who examined the effects of various physical stimuli on the behaviour and mortality of larval *Terranova* (= *Pseudoterranova*) *decipiens*. The stimuli included temperature, electromagnetic radiation, electric current, hydrostatic pressure and modified atmospheres. All stimuli were tested both on 'free' nematodes (in air or physiological saline) and on nematodes that had been 'seeded' back into cod muscle. In general his results highlighted how difficult it could be to kill the nematodes, particularly when they were in the cod muscle.

Temperature was shown to be important in influencing mortality rates within cod muscle, the rate of penetration into the muscle and motility rates along a temperature gradient. Within the cod muscle, 100% mortality occurred within 20 hours at temperatures between -70°C and -25°C. Longer times were required to achieve 100% mortality as the temperature increased towards 0°C. The optimal survival rate of *T. decipiens* within the muscle was achieved at 0°C, although longevity was increased generally between -2.5°C and 7.5°C. Thereafter, mortality increased from 7.5°C to 25°C. Viability increased sharply again at 35°C (between 33-37°C) before decreasing to 70°C. Above 40°C, survival times fell below 6 hours within cod muscle, dropping to only a few minutes at 70°C.

Similar results have been produced by other studies (e.g. Gustafson, 1953, Deardorff *et al.*, 1984), and temperature treatments currently form the basis of the advice from both the EU and FAO regarding consumer health. The survival peaks are likely

related to the lifecycle of the parasite, as they correspond to the temperature range it would encounter in the invertebrate and fish hosts (-2.5°C to 7.5°C) and in the mammalian seal host (33°C - 37°C).

Ronald (1960) also reported a clear thermotactic response in the 'free' nematodes which moved towards temperatures up to 35°C. Increasing the temperature of the cod fillets for a period may therefore increase the motility of the nematodes and encourage them to migrate out of the muscle.

In addition to temperature treatments, Ronald (1962) found that subjecting infected cod muscle to a negative hydrostatic pressure (near vacuum) at 25°C caused mortality of all parasites within 78 hours of treatment. When subjected to high pressure (up to 10bar (1 MPa)), there was no increase in mortality.

Since Ronald's trial was carried out, technological advancements have led to the commercial development of better and more powerful hyperbaric equipment. High pressure treatments are now becoming increasingly popular with the food processing industry as a means of destroying various spoilage bacteria and increasing the shelf life of fresh produce. Recent studies on *Anisakis simplex* show that it can be killed in fish at pressures between 140MPa and 300MPa (Molina-Garcia and Sanz, 2002) over different timescales. Treatment times could be decreased with increasing pressure. Dong *et al.* (2003) found that a treatment of 180 seconds at 207MPa was sufficient to kill 100% of the larvae in king salmon (*Oncorhynchus tshawytscha*) and arrowtooth flounder (*Atheresthes stomias*). No recent studies have been carried out on *Pseudoterranova decipiens*.

Ronald (1962) also examined the effects of different gas treatments on the mortality of *Terranova decipiens*. He found no effects of hydrogen, oxygen or methane gases on mortality compared to controls, whereas nitrogen and carbon dioxide slightly increased survival. Chlorine gas and solutions were able to achieve 100% mortality but current EC regulations state that chlorine can no longer be used as a food preservative (Regulation EC No 2032/2003).

It has been suggested more recently that exposure to different gases may affect the coiling behaviour of *Anisakis simplex* if not the mortality. Specifically, Panebianco *et al.* (2000) reported that CO<sub>2</sub> may have encouraged the nematodes to uncoil, while O<sub>2</sub> encouraged coiling within the body cavity of *Lepidopus caudatus* (silver scabbardfish). It was noted however that this result may have been a result of the different timescales employed in each treatment. However, such manipulation could encourage the nematodes to migrate and make them easier to see during candling, which would be of use to the seafood industry.

Ronald (1963) studied the effects of electromagnetic radiation and electrical current with limited success. He found that 100% mortality could be achieved in 'free' nematodes that were exposed to ultrasound or certain wavelengths of light. These treatments were all ineffective when the nematodes were seeded into the cod flesh however. There was no evidence of any phototactic behaviour, perhaps because this is an unnecessary response in the parasite's natural environment. A range of electric currents were also tested, but had no effect on the parasites within the fish. However, other species of nematode have been shown to be susceptible to electricity (Caveness and Caveness, 1970) and this will be tested on both *A. simplex* and *P. decipiens*.

There is anecdotal evidence from MFG (Linda Wood, *pers. comm.*) that nematodes present in the cod flesh may migrate to the surface over time, allowing them to be more easily seen during candling. Whether this behaviour can be manipulated over a suitable timescale was one of the main aims of this trial. Alternatively, it may be possible to destroy the nematodes *in situ* without damaging the cod fillets.

This trial will examine the effects of some of these stimuli on both *Pseudoterranova decipiens* and *Anisakis simplex*. No such behavioural study has been carried out for the latter. These stimuli were examined using modern equipment and compared between the two parasite species under conditions that can be easily replicated by MFG. Similarly, most of the trials examined parasites that are encapsulated within cod fillets or have been seeded into them, as these are the nematodes MFG are most concerned with.

This trial investigated the effects of:

1. **Light:** To determine the effects of the presence or absence of light on the movement of nematodes in (a) trimmed and (b) untrimmed fillets.
2. **Dehydration:** To determine whether desiccation could have influenced the results of the 'light' test.
3. **Temperature:** To establish the rate of movement of *P. decipiens* and *A. simplex* between 5°C and 30°C. To establish the effects of different temperature treatments on migration rates within fillets were also assessed.
4. **Modified Atmosphere Packing:** To examine the effects of modified atmosphere packing over 48 hours with regard to the migration of the nematodes through the cod fillets.
5. **Electricity:** To determine whether it is possible to kill nematodes within cod fillets using *Crustastun* commercial equipment.
6. **High Pressure Treatment:** To establish the ability of different pressure treatments to kill both *A. simplex* and *P. decipiens* in cod flesh.

## **4.2 Effects of Light**

### **4.2.1 Aims**

The aim of this experiment was to determine whether the migration of anisakid nematodes through the musculature of *Gadus morhua* fillets was affected by the presence or absence of light. A secondary aim was to determine whether trimmed (bones and abdominal flaps removed during processing) or untrimmed (intact) fillets were more suitable for experimental work of this nature.

### **4.2.2 Methods**

#### ***4.2.2.1 Experimental Design***

The cod were caught off the west coast of Iceland using long-lines and were processed at Tros within 24 hours of capture. Processing involved the decapitation, filleting, skinning and trimming (where appropriate) of the cod. Bleeding and gutting were carried out on board the fishing vessels. No further alterations were made to the fillets after arrival at Glasgow University.

The samples arrived in Scotland approximately 48 hours after capture, and were transferred to Glasgow University where they were stored overnight at approximately 3°C, in insulated boxes in the dark. The experiment was carried out in a cold room, at temperatures between approximately 1°C and 5°C. The room was lit by fluorescent lighting in the ceiling.

The experiment began the following day, approximately 72 hours after capture. The samples included 20 trimmed and 20 untrimmed fillets, which were divided into 'light' and 'dark' treatment groups, each containing 10 fillets with naturally encysted nematode larvae. Those fillets exposed to light were laid out on top of black plastic sheets on the open bench with the outside surface facing upwards (Fig. 28). Those kept in the dark were laid out in the same way as the 'light' group, but were placed in crates or on shelves and covered with several black plastic sheets to block out any light.

(a)



(b)



*Figure 28:* Experimental setup of the ‘light’ treatment groups, showing (a) trimmed and (b) untrimmed fillets.

To avoid bias and minimise any difference in the sampling times between treatment groups, the trimmed fillets were sampled before the untrimmed fillets, and were assigned to a treatment group in the order they were removed from the box. Every second fillet was assigned to the ‘dark’ treatment group, and every other fillet to the ‘light’ group. All fillets were candled immediately after being assigned to the appropriate group, and any visible nematodes were marked using plastic pins (laid on the surface of the fillet to avoid causing physical damage to either the fillets or the nematodes). This initial examination was considered to have occurred at 0 hours, and subsequent times were based on this. The fillets were examined in the same order for the duration of the study, to ensure comparisons over the time period were valid. Examinations were carried out after 0, 3, 6, 9, 12, 24, 48 and 72 hours, and both sides of all fillets were photographed each time. Any nematodes that migrated out of a fillet were no longer labelled in the photographs, but were added to the subsequent total counts.

Exposure of the ‘dark’ fillets to some light was unavoidable, as they had to be examined at each time interval and there were insufficient resources to carry out the study using seven different sample sets. However, the time spent in the light was minimized to between one and two minutes at each time interval by removing fillets individually and replacing them immediately after examination.

Following the final observations at 72 hours, the fillets were candled again and all visible nematodes were removed and checked against the previous photographs. This confirmed whether the marks previously recorded did actually identify parasitic nematodes and were not simply marks in the flesh (for example caused by blood spots, bones or areas of skin or membrane that had not been completely removed in processing). In the thinner parts of the fillets (particularly in the abdominal flaps), nematodes were often visible on both sides of the fillet and were subsequently marked twice. These duplicate counts were also corrected at this stage.

Finally, the fillets were cut lengthwise into strips approximately 5-10mm wide and examined carefully for any remaining nematodes that had been missed during the experiment. This final stage produced an accurate count and distribution map of the total nematode burden in each fillet. After removal, the nematodes were fixed and identified according to the procedure described in Chapter 2.

#### ***4.2.2.2 Data Analysis***

The data analysis was carried out using Minitab 13 software. To analyse and compare the effects of each treatment over time, a repeated-measures analysis of variance (ANOVA) test was used through the general linear model (GLM) facility. In this case, although variances were equal ( $p > 0.05$ ), the residuals of the data were not normally distributed. Square-root transformation improved the data, but did not normalise them. ANOVAs are relatively robust to non-normal data if the sample sizes are equal (as they are in this case) (Dr. Philip Smith, *pers. comm.*), but the results should nonetheless be viewed with caution.

Kruskal-Wallis analyses were performed to determine where any differences between treatments existed. 95% confidence was considered significant in all analyses.

### 4.2.3 Results

#### 4.2.3.1 *Effects of light over time*

The repeated-measures ANOVA showed a significant interaction between the treatment groups and time ( $F = 2.97$ ,  $p = 0.000$ ). Time had a weaker effect ( $F = 2.56$ ,  $p = 0.012$ ) than treatment ( $F = 16.32$ ,  $p = 0.000$ ). Kruskal-Wallis analysis within the treatments found the difference was between trimmed and untrimmed fillets. The presence or absence of light had no significant effect. The numbers of visible nematodes in each treatment group over time are shown in Figure 29.

There were significantly more nematodes present in the untrimmed fillets than the trimmed fillets in both treatment groups ( $p < 0.002$ ). This supports the previous data regarding the distribution and numbers of the nematodes in the fillets found in this study.

A total of six nematodes were missed during normal candling (one from the 'untrimmed, light' group and five from the 'untrimmed, dark' group) and were only detected at the end of the experiment. The percentage of nematodes visible in each group at any time is shown in Figure 30, where 100% is the total number of nematodes found at the end of the study.

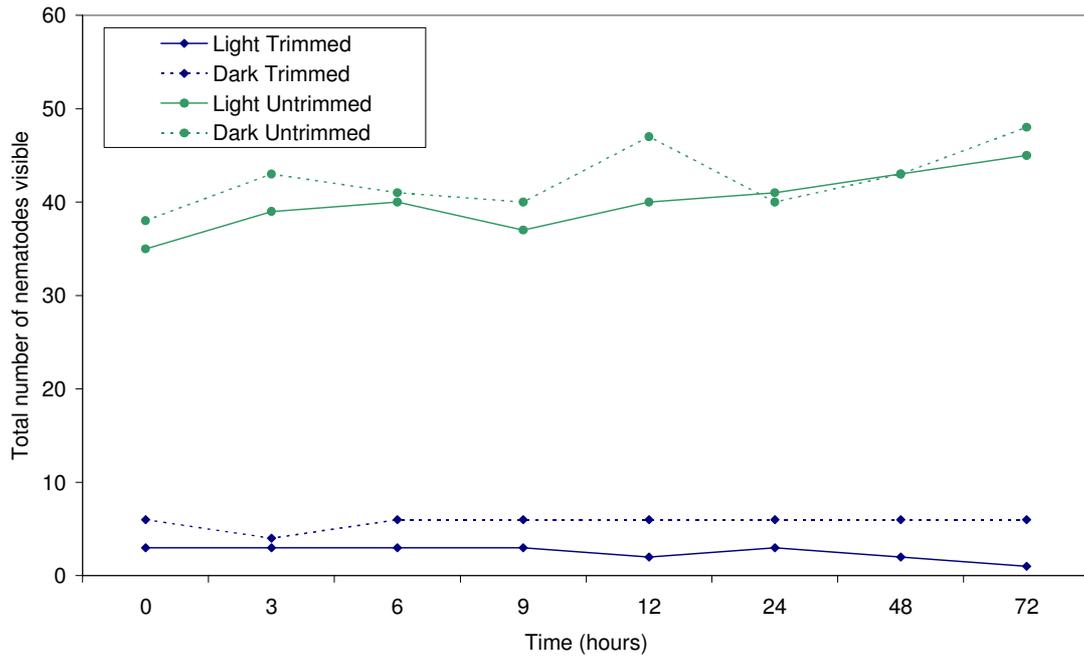


Figure 29: Graph showing the total number of nematodes visible in each treatment group at each time interval. Blue lines show the trimmed fillets; green lines show the untrimmed fillets. The solid lines indicate the 'light' treatments; dotted lines indicate the 'dark' treatments.

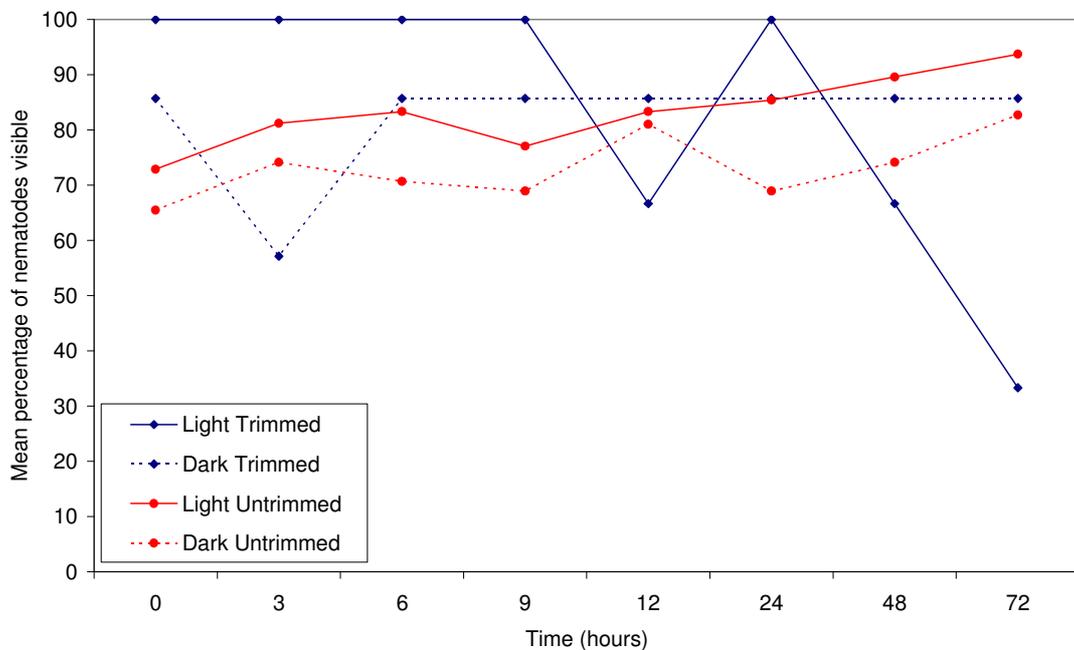


Figure 30: The mean percentage of nematodes visible in each treatment group over time. 100% is the total number of nematodes found by the end of the study.

#### 4.2.3.2 Identification and distribution

The total numbers and identities of the nematodes in each treatment group are shown in Table 10.

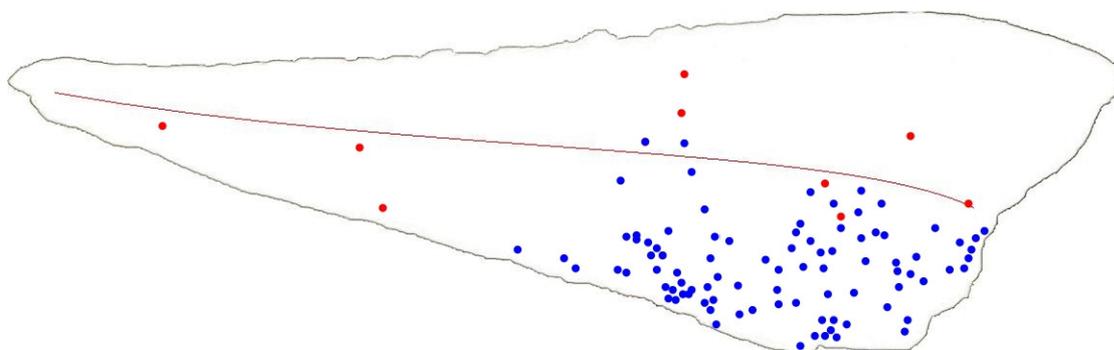
Table 10: Total numbers of each nematode species recovered from the treatment groups.

Treatment Group	Species	Number
Light Trimmed	Unknown	3
Dark Trimmed	<i>Anisakis simplex</i>	1
	<i>Pseudoterranova decipiens</i>	1
	<i>P. decipiens</i> or	3
	<i>Hysterothylacium aduncum</i>	
	Unknown	1
Light Untrimmed	<i>Anisakis simplex</i>	37
	<i>Pseudoterranova decipiens</i>	7
	<i>Hysterothylacium aduncum</i>	1
	Unknown	2
Dark Untrimmed	<i>Anisakis simplex</i>	48
	<i>Pseudoterranova decipiens</i>	2

The approximate distribution of all visible nematodes ( $n = 99$ ) from the untrimmed fillets (from both ‘light’ and ‘dark’ treatment groups) is shown in Figure 31. Only nematodes visible by candling have been included as these were the positions that were recorded photographically. Figure 30 therefore includes three nematode fragments, but no nematodes from deep within the musculature.

*Anisakis simplex* appears to cluster around the abdominal flaps in the epaxial musculature which supports the data previously collected. *Pseudoterranova decipiens* appeared to be more evenly spread throughout the fillet, but numbers were too low to confirm this. The single *Hysterothylacium aduncum* and two ‘unknown’ nematodes

were included with *A. simplex* as their positions could not be differentiated from the photographs. This will again introduce a source of error, but should not be significant given the low numbers involved.



*Figure 31:* Diagram showing the approximate distributions of all visible nematodes in an untrimmed fillet from this trial. The blue dots indicate the positions of *Anisakis simplex*, *Hysterothylacium aduncum* and the ‘unknown’ nematodes. *Pseudoterranova decipiens* is shown in red. The red line shows the horizontal septum that separates the epaxial and hypaxial muscles.

#### 4.2.4 Discussion

The results suggest that fluorescent light has no significant or useful effect on the migration or mortality of the parasites. Ronald (1963) found no effects of light on *Terranova decipiens* when they were coiled within the cod muscle, which is most likely linked to their lifestyle. In their natural environment, neither *P. decipiens* nor *A. simplex* would need to respond to light, as they spend virtually all their lives within host organisms. There is a free-living stage (either L2 or L3) in both species, but there is no evidence that they are sensitive to light. Changes in the apparent visibility of the parasites over time is likely related to a third factor, such as physical changes in the cod fillets or observer error, rather than any movement of the parasites themselves.

Only two nematodes were seen to move during the course of this study. These were relatively large *Pseudoterranova decipiens*, and were both found at the surface of the fillets. It is possible that their movement was stimulated mechanically during processing at Tros or during transport. All the other parasites observed appeared to

remain coiled and motionless throughout, although this will be specifically examined in a later experiment.

The sample size in this test was relatively small, and the total nematode numbers were dominated by *A. simplex*. Further work using larger sample sizes in a variety of lighting conditions may be beneficial to confirm the trends seen here and to provide additional data on *P. decipiens*.

The temperature in the cold room varied over the course of the experiment by approximately 4°C. Although this temperature was maintained within the limits to which MFG works, such low temperatures could have affected the mobility of the nematodes. The mobility of *Pseudoterranova decipiens* and *Anisakis simplex* over a range of temperatures will be assessed in a later experiment.

Desiccation was apparent in all fillets, and although the water content of the fillets was not quantified, those in the 'light' treatments appeared to dry out more than those in the 'dark' treatments. This is probably a result of the fillets in the 'light' group being left uncovered on the bench, whereas the 'dark' group were covered. The effects of this will be examined in a later experiment.

The fillets were approximately three days old by the start of the experiment, meaning that early migrations through the fillet could be missed. However, this time delay is equivalent to the time taken to transport the fillets from Iceland, and the fillets are therefore comparable to those that would be seen by MFG. Additionally, since the majority of the nematodes removed from the fillets were still alive by the end of the trial, this should not have had a significant effect on the potential of the nematodes to migrate.

Finally, in order to candle and photograph the fillets at each time period, it was necessary to expose them to light. Every attempt was made to minimise the length of exposure to the 'dark' fillets, which were examined for between approximately one and two minutes before being returned to the dark. There was insufficient material to analyse different sample sets at each time interval.

### **4.3 Effects of Desiccation**

#### **4.3.1 Aims**

Although not measured quantitatively at the time, dehydration of the fillets appeared to occur during the 'light' experiment, particularly in the uncovered samples. The aim of this experiment was to determine whether the partial desiccation of cod fillets over time could have influenced the movement of anisakid nematodes or their visibility within the fillet during the previous experiment.

#### **4.3.2 Methods**

##### ***4.3.2.1 Experimental Design***

The same procedures were followed as those in the 'light' trial using 18 untrimmed cod fillets. The samples were subjected to the same processing and handling treatment as those previously, over a similar timescale. The temperature in the cold room was more variable during this trial due to mechanical failure, but was still maintained between approximately 2.5°C and 8.5°C. Temperatures in the room were recorded using a data logger.

The experiment began approximately 72 hours after capture. The samples were divided into 'covered' and 'uncovered' treatment groups, each containing nine fillets with naturally encysted nematode larvae. Untrimmed fillets were used to maximise the number of nematodes present in each fillet. The 'uncovered' fillets were laid out in the same way as the 'light' treatment group described in the previous experiment. The covered fillets were covered loosely in cling film before being laid out on the bench (Fig. 32). This was designed to minimise evaporation from the fillets without distorting the flesh.

The distribution of the nematodes was recorded in the same way as during the 'light' trial. All fillets were candled and weighed immediately after being assigned to the appropriate group. The rates of desiccation were estimated by comparing the wet weight of each fillet over time.

(a)



(b)



Figure 32: Experimental setup of the treatment groups, showing (a) uncovered and (b) covered fillets.

#### 4.3.2.2 Data Analysis

The data were analysed using Minitab 13 software. To analyse the effects of each treatment over time, a repeated-measures analysis of variance (ANOVA) test was used through the general linear model (GLM) facility. Variances were equal ( $p > 0.05$ ), but the residuals of the data were not normally distributed. Square root transformation improved the data, but did not normalise them, so the data should again be viewed with caution. 95% confidence was considered significant in all tests.

### 4.3.3 Results

#### 4.3.3.1 Effects of desiccation over time

The results of the repeated-measures ANOVA showed there was no significant interaction between the treatment group and time ( $p = 0.745$ ). There was a significant effect of time ( $p = 0.005$ ), with the number of nematodes observed appearing to decrease over time (Fig. 33).

There was a significant difference in the mean weight lost in each treatment group (Kruskal-Wallis Analysis- Time:  $p = 0.000$ ; Treatment:  $p = 0.000$ ). The data were analysed using Kruskal-Wallis analysis on each of the variables as a two-way ANOVA was inappropriate. The mean weight of the covered fillets was 586.2g at the start; that of the uncovered fillets was 531.8g. After 72 hours, the mean weights had decreased to 563.4g and 423.8g respectively, which were 97% and 81.3% of the starting weights (Fig. 34).

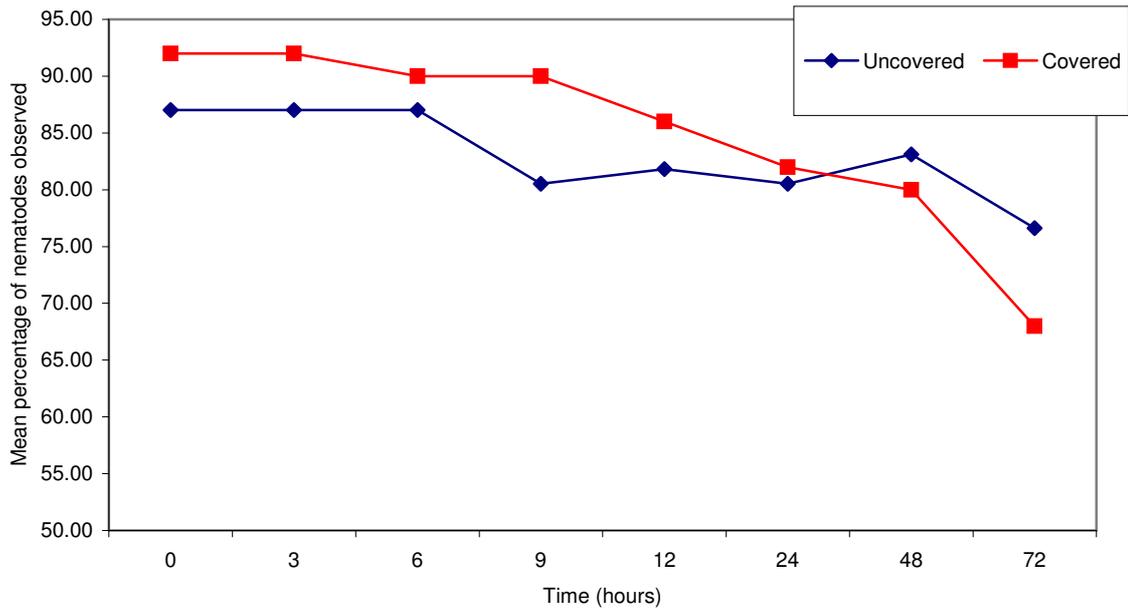


Figure 33: Mean percentage of nematodes visible in each treatment group over time. Repeated measures ANOVA: Treatment vs. time:  $F = 0.61$ ,  $p = 0.745$ ; Time:  $F = 3.1$ ,  $p = 0.005$

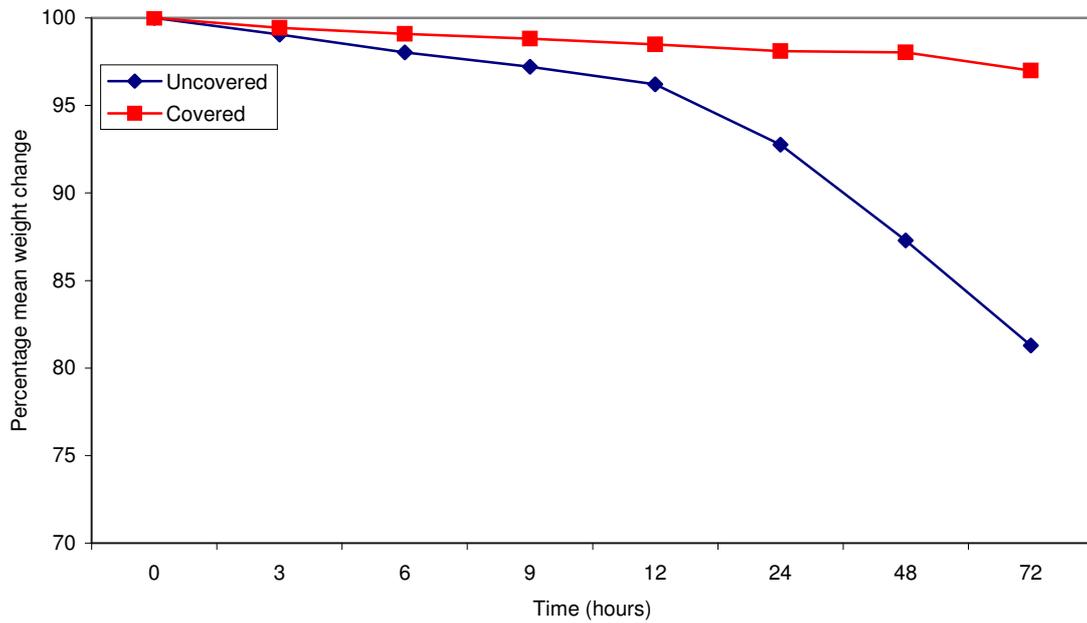


Figure 34: Mean percentage weight change over time in each treatment group. Kruskal-Wallis Analysis – Time:  $H = 33.32$ ,  $df = 6$ ,  $p = 0.000$ ; Treatment:  $H = 60.34$ ,  $df = 1$ ,  $p = 0.000$ .

The rate of weight loss was relatively constant over time in the uncovered group (0.23-0.33g per hour), and higher than the covered group (0.00-0.11g per hour). This suggests that the covered group lost relatively little moisture to the atmosphere, and that most weight was probably lost while the fillets were being handled.

#### 4.3.3.2 Identification

Table 11 shows the numbers and identities of the parasitic nematodes from each fillet.

Table 11: Total numbers of each nematode species recovered from the treatment groups.

Treatment Group	Species	Number
Covered	<i>Anisakis simplex</i>	17
	<i>Pseudoterranova decipiens</i>	1
	Unknown	13
Uncovered	<i>Anisakis simplex</i>	32
	<i>Pseudoterranova decipiens</i>	2
	Unknown	18

Many of the nematodes from these samples were in poor condition, and many were unidentifiable as a result. The distribution data were therefore not plotted as this could not be done accurately.

#### 4.3.4 Discussion

Anisakid nematodes are not tolerant of desiccation (Ronald, 1962, Hauksson, 1992) and so desiccation of the cod musculature in which they are encapsulated may be expected to increase the mortality rate of the parasites. It is also possible that as the flesh dries and toughens, it may become more difficult for the parasites to move through the tissue and migrate.

The results of this trial provided no evidence that desiccation affected the visibility of the nematodes in the flesh, and is therefore unlikely to have affected the results of the 'light' trial. Ronald (1962) found similar results in that decreasing the relative

humidity caused an increase in the mortality rates of 'free' *T. decipiens*, but did not affect those within the cod muscle.

The 'covered' fillets were necessarily uncovered when they were examined, which could have had a small effect on the results. Certainly, if handling caused a small loss of weight each time, it could explain why the wet weight appeared to decrease relatively constantly with each examination, rather than correlating with the amount of time that had passed.

The temperature fluctuated by approximately 6°C over the course of the experiment due to a malfunction in the cooling unit. This could have affected the movement of the nematodes in the flesh, as they are known to be sensitive to temperature (Ronald, 1960). There was no clear evidence that the behaviour in this trial differed to the previous one, but this conclusion should be viewed with caution.

As in the light trial, all nematodes appeared to remain coiled and motionless for the duration of the experiment, and any changes in the numbers of nematodes recorded at any time are likely to be a result of a third factor that was not measured (e.g. observer error).

The sample size in this test was again relatively small, and the nematodes were dominated by *A. simplex*. Further work using larger sample sizes may be beneficial to confirm the trends seen here, but would be unlikely to yield a commercially useful solution.

## 4.4 Effects of Temperature

### 4.4.1 Aims

The aim of this experiment was to determine the effects of temperature (5°C - 30°C) on the motility and migration rates of both 'free' and encysted *Pseudoterranova decipiens* and *Anisakis simplex*.

### 4.4.2 Methods

#### 4.4.2.1 Source of material

For this experiment, live nematodes were taken from factory samples and the abdominal flaps of cod fillets used in previous experiments. The nematodes were identified by their gross morphology at this stage, but were later fixed and their identification confirmed with whole-mount light microscopy as described in Chapter 2.

#### 4.4.2.2 Motility

The motility of the nematodes was estimated using video analysis. The nematodes were checked to ensure they were alive both before and after the temperature treatment. This was done by stimulating each nematode mechanically and watching for a response. If the nematode did not move, it was not used in the experiment.

Motility was measured at five different temperatures, between 5°C and 30°C as shown in Table 12. Each nematode was used only once.

Table 12: Numbers of nematodes recorded at each temperature.

Temperature (°C)	No. <i>Pseudoterranova decipiens</i> examined	No. <i>Anisakis simplex</i> examined
5	4	5
10	5	6
15	6	6
20	6	6
30	6	6

The nematodes were then placed in individual pre-heated Petri dishes containing agar (with 1% NaCl) and approximately 5ml of 1% saline solution. This prevented the nematodes from drying out, and enabled them to move freely. The dishes were then placed in an incubator at the appropriate temperature for at least 10 minutes to allow the nematodes to acclimatise. Each dish was then removed from the incubator in turn, and the nematode was immediately filmed for one minute. Due to the presence of saline and agar in the Petri dish, no significant temperature change would be expected in this time.

The rate of movement was estimated by placing a sheet of acetate over the television screen and marking the position of the head every second. The distance travelled per second was then measured, allowing the velocity of each nematode to be calculated, although no measure was made for total body movement.

#### ***4.4.2.3 Migration and Uncoiling rates***

There has been no clear evidence so far in this study that the nematodes migrate through cod muscle. The majority of parasites recovered from the musculature have been found coiled and motionless (although alive). The aim of this experiment was therefore to test whether temperature can stimulate the uncoiling and subsequent movement of nematodes through the musculature, and over what time scale.

The tests were carried out using the abdominal flaps of 10 cod fillets at each temperature. The fillets were initially candled to confirm the presence of parasites in the flesh, and any uncoiled nematodes on the surface were removed. The fillets were then left for 24 hours at 5°C, or for two hours at 10 or 20°C. During the two-hour trials, the fillets were inspected after 30 minutes, one hour and two hours. Different fillets were used in each temperature trial. The abdominal flaps were used to maximise the numbers of nematodes present in the study.

Nematodes were recorded as 'tightly coiled', 'loosely coiled' or 'uncoiled'. 'Tightly coiled' nematodes were coiled into a circle, and neither the anterior or posterior ends were visible. In 'loosely coiled' nematodes, most of the body was coiled but one end

(assumed to be the anterior end) was visible and uncoiled. Both ends of the nematode were visible in 'uncoiled' nematodes.

At the end of each trial, the fillets were cut into strips as described in Chapter 2 and candled. The numbers of coiled and uncoiled nematodes were recorded, along with any nematodes that had migrated out of the flesh. The nematodes were also recorded as alive or dead, based on whether they moved in response to a mechanical stimulus or immersion in distilled water.

#### ***4.4.2.4 Data Analysis***

The data were analysed using Minitab 13 software. Kruskal-Wallis analysis was used to compare motility at each temperature. The chi-squared test was used to compare the mortality rates over time.

### **4.4.3 Results**

#### ***4.4.3.1 Motility***

The mean speed of each nematode was calculated and compared between temperatures. Kruskal-Wallis analysis showed no significant difference in the rates of movement at different temperatures for either *Anisakis simplex* ( $p = 0.402$ ) or *Pseudoterranova decipiens* ( $p = 0.105$ ) (Fig. 35).

There was no significant difference in the mortality rates between groups ( $p = 0.921$ ).

All specimens were identified as belonging to the correct species.

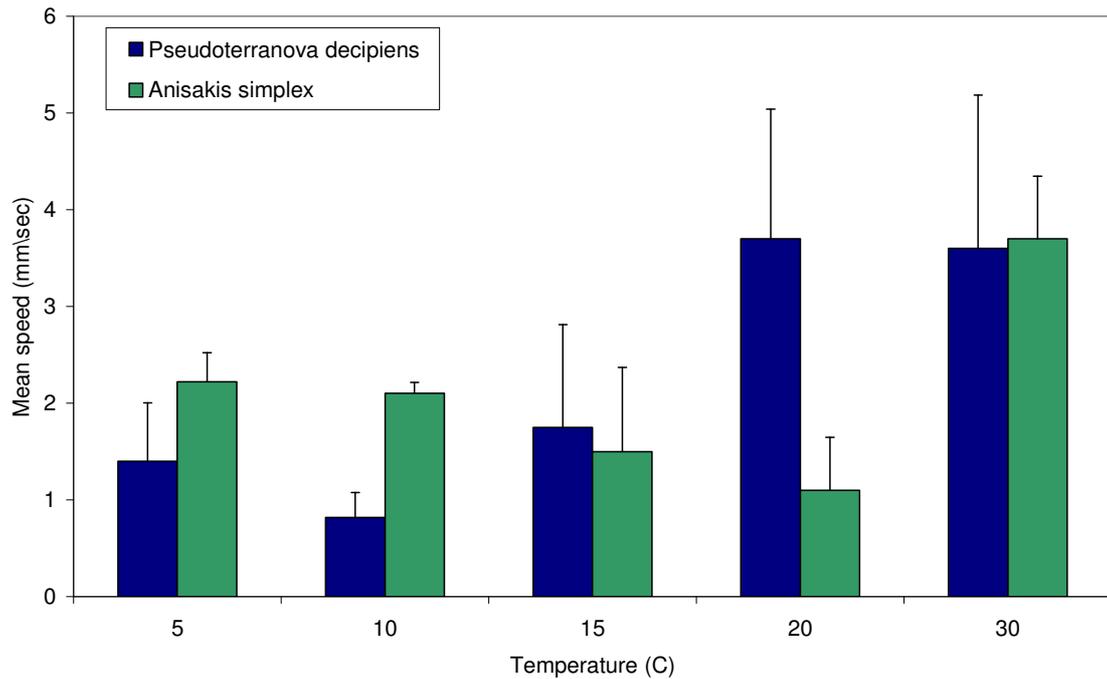


Figure 35: Mean speeds of *Anisakis simplex* and *Pseudoterranova decipiens* at different temperatures. *Anisakis simplex*:  $H = 4.03$ ,  $df = 4$ ,  $p = 0.402$ ; *Pseudoterranova decipiens*:  $H = 7.66$ ,  $df = 4$ ,  $p = 0.105$ . Error bars show one standard error.

#### 4.4.3.2 Migration and Uncoiling

Chi-squared analysis showed no significant difference in the numbers of tightly coiled and loosely coiled nematodes in each temperature treatment ( $p = 0.225$ ). Because there were so few uncoiled nematodes (two after 24 hours at 5°C) their inclusion in the data set invalidated the chi-squared analysis, and they were left out. The numbers of nematodes and their state at the end of each treatment are shown in Table 13.

Table 13: Total numbers of nematodes and their state at the end of each treatment.

Temperature (°C)	No. nematodes	% Alive	% Uncoiled	% Loosely Coiled	% Tightly Coiled
5	114	92.1	0	4.8	95.2
10	40	100	0	2.5	97.5
20	47	100	0	10.6	89.4

#### 4.4.3.3 Identification

The total numbers and identities of the nematodes in each group are shown in Table 14. The ‘unknown’ nematodes from the 5°C trial were most likely all damaged specimens of *A. simplex*, but this cannot be confirmed.

Table 14: Total numbers of nematodes in each temperature treatment.

Treatment Group	Species	Number
5°C for 24 hours	<i>Anisakis simplex</i>	84
	<i>Pseudoterranova decipiens</i>	1
	Unknown	14
10°C for 2 hours	<i>Anisakis simplex</i>	29
	<i>Pseudoterranova decipiens</i>	1
20°C for 2 hours	<i>Anisakis simplex</i>	44
	<i>Pseudoterranova decipiens</i>	2

#### 4.4.4 Discussion

##### 4.4.4.1 Motility

The results suggest that temperature has no effect on the rates of movement of either *A. simplex* or *P. decipiens*. This contradicts the results reported by Ronald (1960), who found a linear relationship between motility and temperature in *T. decipiens*. Indeed, a linear relationship would be expected in poikilothermic organisms. The lack of such a relationship could be explained by the relatively low sample sizes in the present study and high variability between individual specimens (Ronald (1960) used at least 1000 specimens in each of his trials). Unfortunately, there was insufficient time and specimens to expand the sample sizes here, but further work would be extremely useful.

The experimental conditions may have influenced the behavioural response of the nematodes. For example, moving the Petri dishes from the incubator to the camera could have mechanically stimulated the nematodes and biased the results. Similarly, while selecting nematodes that were visibly mobile ensured that all specimens were alive, it could also have introduced another source of error and biased the results.

#### ***4.4.4.2 Migration and uncoiling rates***

The 5°C treatment cannot be directly compared to the 10°C and 20°C treatments because the durations were different. However, the fact that so few nematodes uncoiled through the trial suggests that a single temperature treatment is insufficient to stimulate migration of the nematodes.

The different time scales used for the different temperature treatments reflect my own observations in previous trials and current practice by MFG. During the ‘Light’ and ‘Desiccation’ trials (Chapters 4.2 and 4.3), there was no indication that the nematodes moved rapidly, if at all at low (<10°C) temperatures within cod muscle. The present trial was designed to test these observations more conclusively at constant temperatures. Based on MFG’s current practice, 24 hours was considered a suitable and practical length of time to leave the fillets. Under current industrial guidelines, cod fillets may be exposed to temperatures up to 10°C for short periods of time, and two hours was considered sufficient to determine whether there was a response. The 20°C trial was included to determine whether higher temperatures could elicit a stronger response from the nematodes, but was not intended to be of practical use.

## **4.5 Modified Atmosphere Packing**

### **4.5.1 Aims**

Modified atmosphere packing (MAP) is used extensively in the seafood industry to preserve various products by inhibiting the growth of certain spoilage bacteria and prolonging shelf-life (e.g. Dalgaard *et al.*, 1993, Soccol and Oetterer, 2003). It has been suggested that MAP may affect the behaviour of encapsulated *A. simplex* and *Pseudoterranova decipiens*, and this will be examined in this trial.

### **4.5.2 Methods**

#### ***4.5.2.1 Experimental design***

Pieces of cod were packed in modified atmospheres using a gas-packing machine (Model PA210 Cap Heat Sealing machine, manufactured by Packaging Automation Ltd) at the University Marine Biological Station Millport (UMBSM). Two pieces were put in each pack. The gas mixes were confirmed by analysing the gases in empty packs with an OXYBABY analyser. This was done twice for each gas mix, and an error rate of  $\pm 5\%$  was considered acceptable.

The initial trial examined the effects of 100% CO<sub>2</sub>, O<sub>2</sub> and N<sub>2</sub> on the nematode parasites within cod fillets after 48 hours. A control group was packed in air. For each treatment, 10 abdominal flaps were packed containing naturally encysted nematode larvae. The abdominal flaps were used to maximise the number of nematodes used in the study, as there were very few nematodes visible in the fillets. Any fillets with visible nematodes were also packaged, but these were not included in the statistical analysis to keep the treatments standardised.

Each piece of cod was examined briefly prior to packing, and the overall appearance of the nematodes in the musculature was briefly described. Any partially coiled or uncoiled nematodes that were visible at this stage were noted but not removed.

The packs were then transferred to Glasgow University and stored for approximately 48 hours at 5°C. After this time, the gas mixtures in each pack were checked using the OXYBABY analyser and the pack weights were recorded. The packs were opened and the portions examined for parasites over a light box. The state (tightly

coiled, loosely coiled or uncoiled; alive or dead) of each nematode was recorded and compared between the treatments.

#### **4.5.2.2 Data Analysis**

The results were analysed using the Scheirer-Ray-Hare extension to the Kruskal-Wallis test. This test is essentially a non-parametric version of a two-way ANOVA, and is carried out on the ranks of the data set. Only the abdominal flap samples were included in the analysis, in order to standardise the data sets.

### **4.5.3 Results**

#### **4.5.3.1 Effects of MAP**

The Scheirer-Ray-Hare test showed that there was a difference in the state of the nematodes ( $p = 0.000$ ) in each group, with more tightly coiled nematodes being present than either loosely coiled or uncoiled nematodes. The state of the nematodes was not affected by the gas present however, and there was no significant interaction between the two variables ( $p = 0.959$ ). These results are shown in Figure 36.

At the end of the trial, between 85.71% ( $N_2$  treatment) and 92.73% (control treatment) of all nematodes were tightly coiled in the cod muscle. Between 0% (control treatment) and 14.29% ( $N_2$  treatment) were loosely coiled and between 0% ( $N_2$  treatment) and 7.27% (control treatment) were uncoiled.

The mean gas mixes at the start and end of this trial are shown in Table 15. Only  $CO_2$  and  $O_2$  levels were measured by the OXYBABY analyser, so  $N_2$  is not included. It can be assumed that  $N_2$  comprises the remainder of the starting mix in each case. Dissolution of  $CO_2$  may have affected the ratio of gases at the end of the trial, so estimates of  $N_2$  at this point should be viewed cautiously.

The  $CO_2$  levels decreased in both the 100%  $CO_2$  and control (air) groups. The percentage decrease in  $CO_2$  was strongly correlated to the mass of cod in each pack ( $R^2 = 0.96$ ).

Table 15: Mean composition of gas mixes at the start and end of each trial.

Trial	Mean % at start		Mean % at end	
	O <sub>2</sub>	CO <sub>2</sub>	O <sub>2</sub>	CO <sub>2</sub>
100% O <sub>2</sub>	100	0.25	96.2	0.9
100% CO <sub>2</sub>	0.4	98.25	1.8	83.8
100% N <sub>2</sub>	0.2	0.6	0.1	2.5
Air	21.4	0.1	2.9	18.1

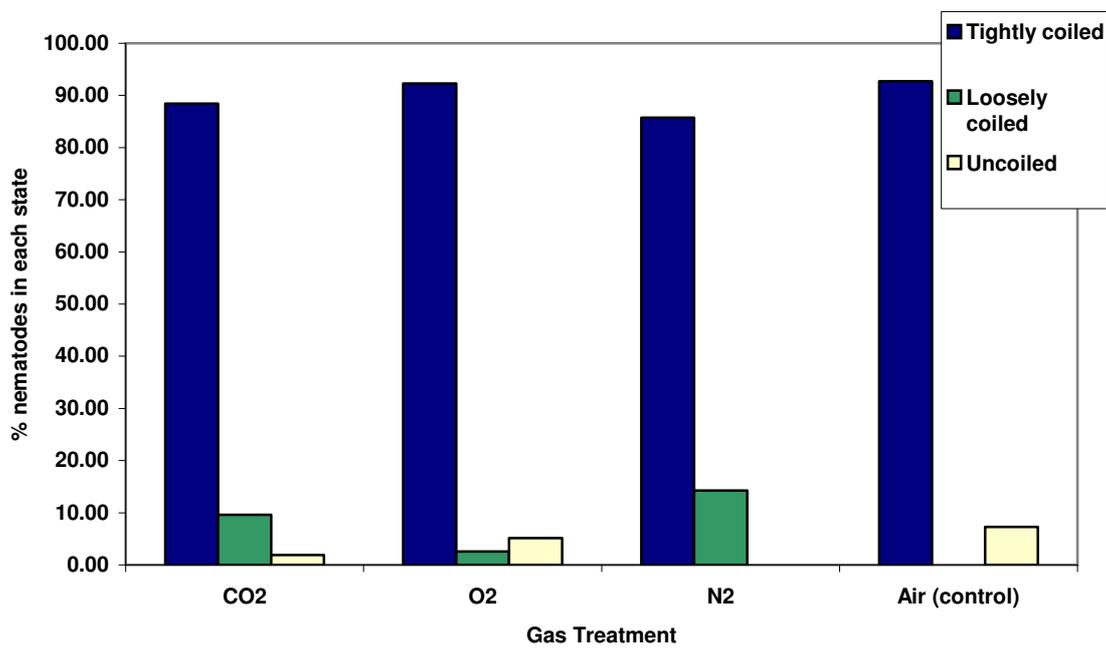


Figure 36: Percentage of nematodes in each state under the four gas treatments.

Schierer-Ray-Hare test:  $H = 115.33$ ,  $df = 2$ ,  $p = 0.000$ .

#### 4.5.3.2 Identification

The total numbers and identities of the nematodes in each group are shown in Table 16.

Table 16: Total numbers of nematodes in each gas treatment.

Treatment Group	Species	Number
Air (Control)	<i>Anisakis simplex</i>	51
	<i>Pseudoterranova decipiens</i>	3
	Unknown	1
100% CO <sub>2</sub>	<i>Anisakis simplex</i>	49
	<i>Pseudoterranova decipiens</i>	4
	Unknown	1
100% N <sub>2</sub>	<i>Anisakis simplex</i>	56
100% O <sub>2</sub>	<i>Anisakis simplex</i>	38
	<i>Pseudoterranova decipiens</i>	1

#### 4.5.4 Discussion

There is no evidence from this trial that MAP had any impact on the motility or mortality of the nematodes in the cod muscle over 48 hours. These results are similar to those reported by Ronald (1962), who found limited effects of gas treatment on the mortality of *T. decipiens*. Panebianco *et al.* (2000) suggested that CO<sub>2</sub> may stimulate uncoiling behaviour in *A. simplex*, but there was no evidence from the current study that any of the gas treatments had a significantly different effect to the control group.

The decrease in CO<sub>2</sub> levels in the 100% CO<sub>2</sub> and control packs is likely due to diffusion of the gas into the cod. Gaseous CO<sub>2</sub> is highly soluble and is known to dissolve and diffuse into foodstuffs under modified-atmosphere conditions (e.g. Sivertsvik *et al.*, 2004).

It was observed that the nematodes in the CO<sub>2</sub> treatment group seemed to have a softer capsule around them than nematodes in the other groups. This observation was not quantified, but could form the basis for further study. Dissolved CO<sub>2</sub> forms carbonic acid and may directly dissolve the nematodes' capsules, or may trigger a biological response. Under natural conditions, these nematodes must excapsulate once in the final host and attach to the stomach wall. Since the stomachs of host animals are warm (approx. 38°C) and acidic, such conditions could potentially 'activate' the nematodes and stimulate movement. While this is purely speculation at this point, it would be worth investigating further to determine the effects over a longer time span, or in conjunction with other treatments (e.g. high temperature) which similarly replicate the conditions found in the mammalian final host, without significantly damaging the cod tissue.

## **4.6 Electrocutation**

### **4.6.1 Aims**

This trial aimed to determine whether *A. simplex* or *P. decipiens* could be destroyed within cod muscle using *Crustastun* commercial equipment.

### **4.6.2 Methods**

#### ***4.6.2.1 Experimental design***

This trial was carried out using a ‘*Crustastun*’ Single-Stunner machine, which has been designed to humanely kill small numbers of crustaceans in commercial restaurants. The live animal is placed on a spring-loaded tray over a bath of salt water (seawater equivalent) and a lid is closed over it. The machine delivers a current of 110v (2-5amps) for a period of 5 seconds for lobsters, or 10 seconds for crabs (*Crustastun* website).

Sections of cod fillet were cut into strips approximately 2cm wide by 5cm long and 1cm deep. Four strips were seeded with five live (motile) specimens of either *Anisakis simplex* or *Pseudoterranova decipiens* (10 of each were used in total) (Figs. 37 & 38). The seeded cod strips were covered with a second strip to form a ‘sandwich’ and were placed in the *Crustastun* machine (Figs. 39 & 40).

The ‘sandwiches’ were subjected to five cycles of electrocution (using the ‘crab’ setting). The effects were assessed by observing the motility of the specimens in saline immediately after each cycle. Any nematodes that were seen to move (either spontaneously or after being gently uncoiled using forceps) were considered to be alive, while those that were not were considered to be dead. The same nematodes were present in each group for the duration of the trial. The temperature at the centre of the cod ‘sandwiches’ was also noted. There was a pause of approximately 10 minutes between each cycle while this was done.

#### ***4.6.2.2 Data Analysis***

The data were analysed using Minitab 13 software. Kruskal-Wallis analysis was used to compare the temperatures of each cod sandwich. No analysis was carried out on the mortality rates as the data do not meet test assumptions.



Figure 37: *A. simplex* on a cod portion. Nematodes are indicated with a red arrow-head.



Figure 38: *P. decipiens* on a cod portion



Figure 39: Cod 'sandwiches' in the *Crustastun* Single stunner



Figure 40: Cod 'sandwiches' in the *Crustastun* Single Stunner (close-up).

### 4.6.3 Results

Electrocution appeared to have very limited effects on the nematodes in the cod. The mortality rates for each group are shown in Table 17. Only one group showed any increase in mortality (*Pseudoterranova* gp. 1) with increasing numbers of cycles. All other specimens survived the treatment with few (if any) apparent ill-effects. The data cannot be analysed statistically as they do not conform to test assumptions, but it is clear that the levels of mortality are not high enough to be commercially useful.

Table 17: Numbers of dead nematodes following each cycle. There were five specimens in each group.

Sample	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5
<i>Anisakis</i> gp. 1	0	0	0	0	0
<i>Anisakis</i> gp. 2	0	0	0	0	0
<i>Pseudoterranova</i> gp. 1	0	0	2	2	3
<i>Pseudoterranova</i> gp. 2	0	0	0	0	0

The temperature of each ‘sandwich’ after each cycle is shown in Figure 41.

Generally, there was a progressive increase in the temperature of the fish as the number of cycles increased (mean increase = 15.7°C after five cycles). The temperature varied significantly with cycle number (Kruskal-Wallis:  $p = 0.011$ ). Post-hoc analysis showed that cycles four and five were significantly hotter than the starting temperature ( $p = 0.0009$  and  $0.0025$  respectively). Burn marks were evident along the edges of the cod pieces that had touched the edges of the tray (Fig. 42).

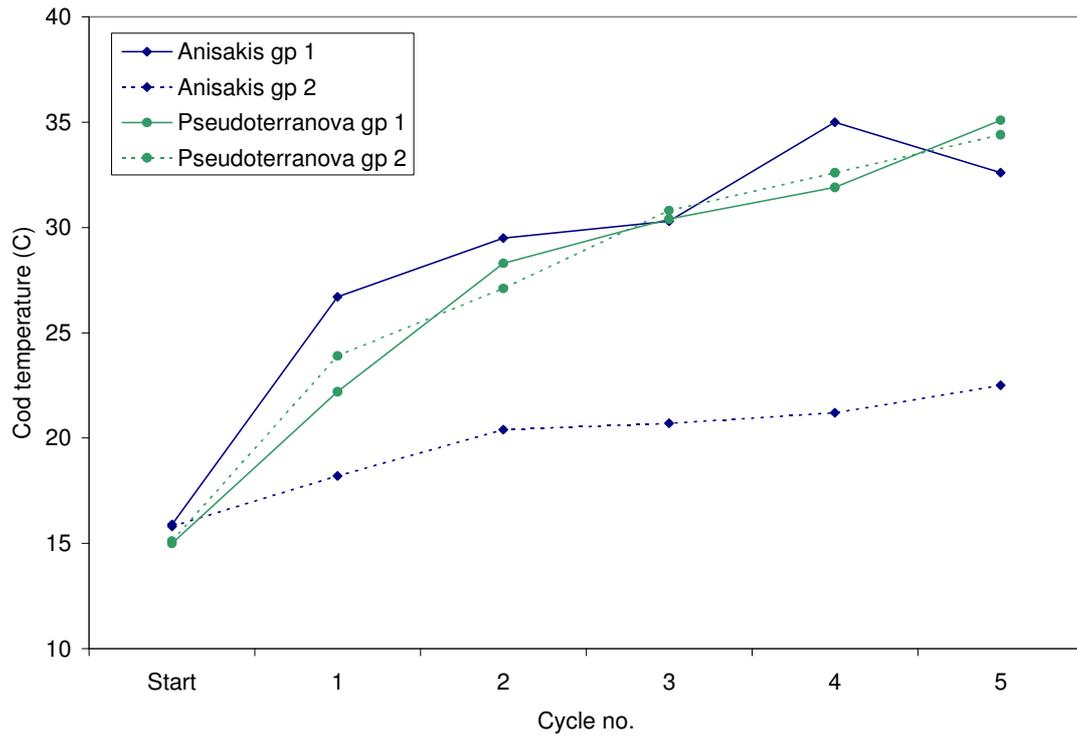


Figure 41: The temperature at the centre of each cod ‘sandwich’ following each cycle.



Figure 42: Burn marks on a cod portion after five treatment cycles.

#### 4.6.4 Discussion

There is no evidence that electrocution of cod pieces using the *Crustastun* is effective in destroying *A. simplex* or *P. decipiens*. This supports the results of Ronald (1962), who found that electrocution had no effect on the mortality of *Terranova decipiens*, despite increasing the current to a maximum of 810v and 55ma (continuous direct current). Similar trials had not previously been carried out on *A. simplex*.

Ronald (1962) hypothesised that the lack of any effect could be related to the structure of the nematode. Within cod flesh, Ronald demonstrated that *T. decipiens* had a higher electrical resistance than the cod flesh. Any current applied to the cod would follow the path of least resistance and would therefore not pass through the nematode at all. Choudhury and Bublitz (1994) confirmed that both *A. simplex* and *P. decipiens* have a higher electrical resistance than cod muscle.

The *Crustastun* had an apparent effect on the quality of the cod flesh. The quality was not formally assessed, but the increase in temperature (mean = 15.7°C) after five treatment cycles is unlikely to be compatible with current processing practice. The apparent whitening of the flesh and scorching along the sides are also unlikely to be acceptable.

## **4.7 High Hydrostatic Pressure (HHP) Treatment**

### **4.7.1 Aims**

High pressure treatment is receiving increasing interest from the seafood processing industry as a means to inactivate spoilage bacteria and increase the shelf life of the produce (e.g. Linton *et al.*, 2003). Previous studies (Molina-Garcia and Sanz, 2002, Dong *et al.*, 2003) on fish have shown that *Anisakis simplex* can be destroyed using high pressure treatments, but no trials have yet been conducted on *Pseudoterranova decipiens*.

### **4.7.2 Methods**

#### ***4.7.2.1 Experimental design***

The effects of high hydrostatic pressure (HHP) treatment were assessed using the high pressure facility at the Agri-Food and Biosciences Institute in Belfast. An initial preliminary trial was carried out using only *A. simplex* and was followed later by a more thorough investigation on both *A. simplex* and *P. decipiens*.

The nematodes were seeded onto pieces of cod fillet which had been cut into portions measuring approximately 5cm by 10cm, and were between 1.5cm and 2cm thick following the ‘fish sandwich’ method described by Molina-Garcia and Sanz (2002). The nature of high hydrostatic pressure is such that the pressure exerted on each part of the ‘sandwich’ and its contents will be the same regardless of orientation or thickness of the cod. Small differences between the cod portions will therefore have no effect, and the results should be comparable to those on encysted nematodes in commercial fillets. The portions were stored on ice or frozen until needed. No encapsulated nematodes were visible in the portions.

#### ***4.7.2.2 Preliminary Trial***

For the preliminary trial, specimens of *A. simplex* were taken from the viscera of dissected fresh herring (*Clupea harengus*) as no nematodes were available from cod at the time of the experiment. Identification was based on the gross morphology of the specimens. Ten nematodes were then seeded onto one cod portion and covered with a second to form a ‘sandwich’ (Fig. 43). Two ‘sandwiches’ were vacuum packed separately and pressurised for 90 seconds at either 150, 180, 200, 220, 240 or 400MPa (Fig. 44). An additional sample was treated at 180MPa for five minutes to assess the

effects of increased duration. A vacuum-packed, non-pressure treated ‘sandwich’ was used as a control.

The effects were assessed by examining the motility of the *A. simplex* in distilled water approximately 1-2 hours after HPP treatment. The nematodes were left in distilled water, and gently uncoiled using forceps. Any nematodes that were then seen to move (beyond simply ‘springing’ back to their previous shape) were considered to be alive, while those that were not were considered to be ‘apparently dead’. Since some recovery could potentially occur over time, ‘apparent mortality’ was recorded rather than ‘actual mortality’.

Samples subjected to 400MPa were then fixed for SEM or sectioning and light microscopy to identify any areas that had been damaged by the pressure treatment.



*Figure 43:* Preparation of the cod ‘sandwiches’.



*Figure 44:* Cod ‘sandwiches’ in pressure chamber after treatment to 200MPa.

#### **4.7.2.3 Main Trial**

The full trial was carried out using specimens of *A. simplex* that had been extracted from the abdominal flaps of cod, and on *P. decipiens* that had been removed during processing at Tros (approximately 48 hours before experimental work began). Identification was based on the gross morphology of the specimens. Ten nematodes (of the same species) were seeded onto cod ‘sandwiches’ as before, and were then exposed to 180, 200, 220 or 240MPa for 90 seconds, 3, 5 or 10 minutes. Due to insufficient material, *A. simplex* was not treated at 240MPa or for the 10 minute treatments. A vacuum-packed, non-pressure treated ‘sandwich’ was used as a control for each species.

Following treatment, the nematodes were placed in a dish of fresh water and watched for movement. Any nematodes displaying spontaneous movement or movement in response to gentle mechanical stimulus were considered to be alive, while those that simply ‘sprang back’ to their original shape or did not move were ‘apparently dead’. This was either done immediately after treatment, or after 24 hours. Due to time constraints at the facility, it was impossible to immediately examine all treatment groups thoroughly, and some delay was unavoidable.

After examination, two nematodes from each treatment group were immediately fixed in 2% gluteraldehyde in 0.1M sodium cacodylate buffer for microscopic examination of the fine internal structure to determine if any internal damage was sustained during treatment. The remaining nematodes were examined again after 72 hours to determine whether any recovery or delayed mortality had occurred, before being fixed in gluteraldehyde and arsenic for sectioning.

#### **4.7.2.4 SEM**

The *A. simplex* samples from the preliminary trial were fixed in 2% p-formaldehyde/ 2.5% gluteraldehyde with 0.1M PO<sub>4</sub> buffer (4% sucrose/1.5% NaCl) for approximately two hours before being transferred to 0.1M PO<sub>4</sub> (8% sucrose) buffer rinse overnight. The specimens from the cod were fixed for approximately 7 hours (as we were travelling) before being transferred to the buffer rinse for the weekend. The samples were stored on ice or in a freezer at 0°C until needed.

The samples were then treated as described in Chapter 2, and examined for any signs of physical damage.

#### **4.7.2.5 Light Microscopy**

After fixation, the specimens were given to Dr. Ian Montgomery for sectioning and mounting. After fixation, the samples were washed in 0.1M sodium cacodylate buffer, then fixed in 1% osmium tetroxide in 0.1M sodium cacodylate for four days. The fixed samples were embedded in Durcapan resin then sectioned with glass knives. Unfortunately the resin did not penetrate the nematodes, and this aspect of the work had to be abandoned.

### 4.7.3 Results

#### 4.7.3.1 Preliminary trial

The apparent mortality rate achieved in each trial is shown in Table 18. A minimum pressure of 220MPa was required to have any apparent effect on mortality following a 90 second treatment. 400MPa was the only treatment causing 100% apparent mortality of *A. simplex* although lower pressures may be equally effective and should be investigated further.

Increasing the treatment time to five minutes appeared to increase the apparent mortality rate at 180MPa compared to the shorter treatment.

*Table 18:* Percentage apparent mortality of *A. simplex* in the cod ‘sandwiches’ after each treatment. The no. of specimens in each treatment group is shown in italics; treatments resulting in 100% apparent mortality are highlighted in bold.

		Duration (mins)	
		1.5	5
<b>Pressure (Mpa)</b>	<b>150</b>	0 <i>(17)</i>	
	<b>180</b>	0 <i>(18)</i>	23.53 <i>(17)</i>
	<b>200</b>	0 <i>(20)</i>	
	<b>220</b>	42.9 <i>(14)</i>	
	<b>240</b>	80 <i>(15)</i>	
	<b>400</b>	<b>100</b> <i>(17)</i>	

Control group = 0% apparent mortality (7 specimens)

### 4.7.3.2 Main Trial

The apparent mortality rate achieved in each trial is shown in Tables 19 (*A. simplex*) and 20 (*P. decipiens*). None of the treatments resulted in 100% apparent mortality of *A. simplex*, and there was no apparent recovery or delayed mortality for this species. Treatments of at least 220MPa for 5 minutes initially appeared to be consistently effective in causing 100% apparent mortality of *P. decipiens*. Some recovery occurred in this species however, and only treatments of 240MPa for at least 3 minutes were 100% effective after 72 hours.

Table 19: Percentage apparent mortality of *A. simplex* (a) immediately after each treatment and (b) after 72 hours. The no. of specimens in each treatment group is shown in italics; treatments resulting in 100% apparent mortality are highlighted in bold.

(a)

		Duration (mins)		
		1.5	3	5
Pressure (Mpa)	180	20 <i>(10)</i>	29 <i>(7)</i>	0 <i>(9)</i>
	200	22 <i>(9)</i>	33 <i>(9)</i>	10 <i>(10)</i>
	220	11 <i>(9)</i>	56 <i>(9)</i>	43 <i>(7)</i>

Control group = 0% apparent mortality (6 specimens)

(b)

		Duration (mins)		
		1.5	3	5
Pressure (Mpa)	180	12 <i>(8)</i>	0 <i>(5)</i>	0 <i>(9)</i>
	200	0 <i>(10)</i>	11 <i>(9)</i>	25 <i>(8)</i>
	220	14 <i>(7)</i>	29 <i>(7)</i>	20 <i>(5)</i>

Control group = 0% apparent mortality (5 specimens)

Table 20: Percentage apparent mortality of *P. decipiens* (a) immediately after each treatment and (b) after 72 hours. The no. of specimens in each treatment group is shown in italics; treatments resulting in 100% apparent mortality are highlighted in bold.

(a)

	Duration (mins)				
	1.5	3	5	10	
Pressure (Mpa)	180	30 <i>(10)</i>	50 <i>(10)</i>	<b>100</b> <i>(10)</i>	80 <i>(10)</i>
	200	30 <i>(10)</i>	90 <i>(10)</i>	<b>100</b> <i>(10)</i>	90 <i>(10)</i>
	220	60 <i>(10)</i>	50 <i>(10)</i>	<b>100</b> <i>(10)</i>	<b>100</b> <i>(10)</i>
	240	<b>100</b> <i>(10)</i>	<b>100</b> <i>(10)</i>	<b>100</b> <i>(10)</i>	<b>100</b> <i>(10)</i>

Control group = 0% apparent mortality (10 specimens)

(b)

	Duration (mins)				
	1.5	3	5	10	
Pressure (Mpa)	180	0 <i>(7)</i>	0 <i>(8)</i>	0 <i>(10)</i>	87 <i>(8)</i>
	200	0 <i>(10)</i>	10 <i>(10)</i>	50 <i>(8)</i>	87 <i>(8)</i>
	220	62 <i>(8)</i>	25 <i>(8)</i>	68 <i>(8)</i>	87 <i>(8)</i>
	240	30 <i>(10)</i>	<b>100</b> <i>(10)</i>	<b>100</b> <i>(8)</i>	<b>100</b> <i>(8)</i>

Control group = 0% apparent mortality (10 specimens)

#### 4.7.3.3 SEM

Observations of *A. simplex* from the preliminary trial under SEM showed no damage to the surface of the cuticle in any of the three treatment groups examined. A representative image is shown in Figure 45. Comparisons to Weerasooriya *et al.* (1986) show that the specimens were all *Anisakis simplex* (L3).

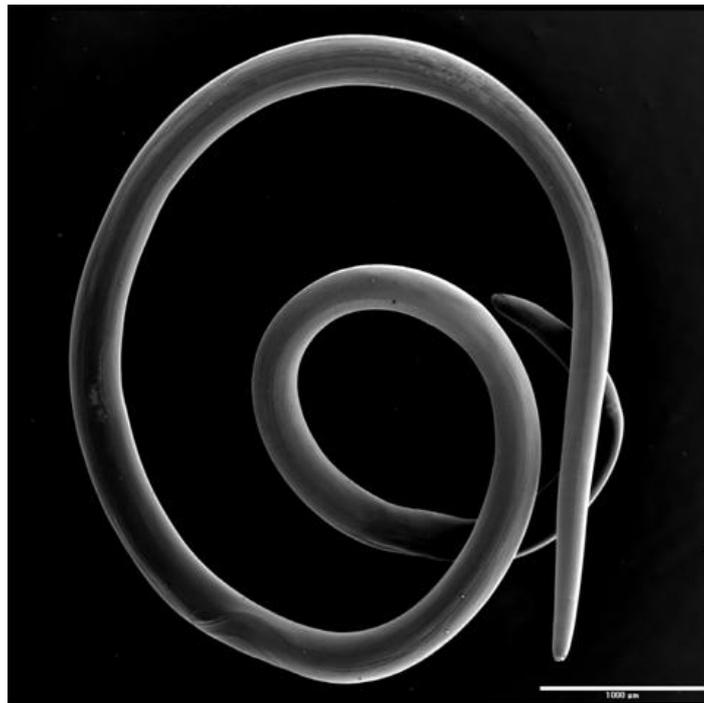


Figure 45: A whole specimen of *A. simplex* after HHP treatment at 400MPa. Scale bar = 1mm.

#### 4.7.3.4 Light Microscopy

No results were obtained from the light microscopy as the techniques caused too much damage to the specimens to yield any useable data.

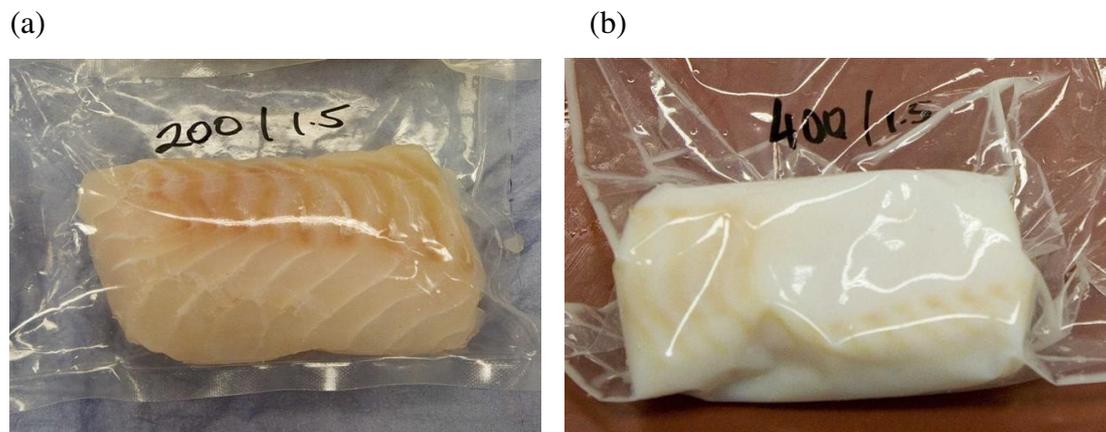
#### 4.7.3.5 Observations on nematode motility

No quantitative measures of motility were made during these trials, although some differences were noted following the HHP treatments. Prior to HHP treatment, the specimens were highly active when placed in distilled water at room temperature and moved rapidly. Pressure-treated nematodes by comparison appeared much more sluggish, and some of them barely moved when stimulated. While it was clear that these nematodes were not dead, it is possible that they may have been disabled in some way by the treatment. The subsequent recovery of several *P. decipiens*

specimens after 72 hours suggests that such effects may only be temporary in this species. Further tests into the motility and infectiveness of such specimens should be carried out to determine whether lower pressure treatments may be sufficient to solve the nematode problem without damaging the cod flesh.

#### **4.7.3.6 Observations on fish quality**

Although no formal assessment was carried out, some changes in the cod flesh were apparent following the HHP treatments. Prior to treatment, the cod portions were moist and soft to the touch and appeared translucent. After all treatments, the texture had altered and the portions felt firmer and more 'rubbery'. They also appeared to be somewhat drier than the original portions. It became increasingly difficult to separate the cod portions as the pressure was increased. The treatment at 400MPa also caused the portions to become opaque and white, and looked superficially like cooked fish. A comparison of portions treated at 200MPa and 400MPa is shown in Figure 46 (colours are accurate). There was no apparent change in the texture or colour of the control sample, which was vacuum-packed but not pressurised.



*Figure 46:* Cod portions after treatment at (a) 200MPa and (b) 400MPa.

#### 4.7.4 Discussion

The results from the main trial suggest that a minimum treatment of 220MPa for 3 minutes is required to achieve 100% apparent mortality of *P. decipiens*. No minimum safe treatment was found for *A. simplex*, although 240MPa and 10 minute treatments were not fully tested. The results also suggest that higher pressures and longer treatment times appear to become progressively more effective at killing *P. decipiens* in the cod muscle. No such trend was apparent for *A. simplex*.

Molina-Garcia and Sanz (2002) and Dong *et al.* (2003) both report that increasing treatment times will increase the mortality rate in *A. simplex* and that treatment times can be decreased by increasing the pressure and vice versa. This is similar to the results found in the present study for *P. decipiens*, but could not be verified for *A. simplex*. This may be a result of the small sample sizes used in the current trial, or may be due to differences between cryptic species.

Molina-Garcia and Sanz (2002) report that a treatment at 200MPa for 10 minutes will kill all *A. simplex*, whereas Dong *et al.* (2003) report a pressure of 207MPa for 3 minutes is sufficient. The present study could not verify whether these treatments are effective. However, given that 220MPa for 5 minutes was found to be ineffective in achieving 100% mortality of *A. simplex*, it is unlikely that shorter treatments at lower pressures would be effective.

However, several factors could have influenced the results of their experiments. Molina-Garcia and Sanz (2002) for example allowed the samples of *A. simplex* to reach room temperature before examination as this reportedly encouraged more rapid movement. Their specimens were only examined for movement for one minute however, which may not have been long enough to allow the nematodes to recover from the treatment and behave as normal. Additionally, the specimens examined were removed from the original fish host and stored at 4°C for up to five days before treatment. Based on my own observations of this species, leaving them for this length of time could have decreased the viability of the specimens and may have made them more susceptible to the high pressure treatment.

Dong *et al.* (2003) examined the nematode specimens after 48 hours, and following HCl-pepsin digestion. The nematodes were left encapsulated in the fish, which avoided possible mechanical damage, but means that the authors could have been subjecting dead nematodes to their HHP trials, thus biasing their results.

The observation that pressure-treated nematodes had reduced apparent motility could be indicative of possible long-term effects on the mortality of the nematodes. The recovery of *P. decipiens* after 72 hours suggests that this may not be the case however, and that the initial sluggishness could simply be a direct, short-term effect of the treatment.

Although there were changes in the numbers of motile *A. simplex* after 72 hours in the full trial, there was no clear pattern of recovery or delayed apparent mortality. It is impossible to conclude whether these changes are related to the treatments or another factor, and further trials with higher sample numbers would be required.

By contrast, *P. decipiens* did appear to show signs of recovery after 72 hours. Of the eight treatment groups which were initially considered 100% effective, five contained motile specimens after 72 hours. There was no evidence of delayed mortality.

High pressure treatments are known to adversely affect the quality of cod flesh. Angsupanich and Ledward (1998) described the effects of different pressure treatments on cod muscle between 0 and 800MPa. By 200MPa, they reported some loss of translucency in the fillets, which became increasingly opaque with increasing pressure and eventually looked similar to cooked fish. Myosin began to degrade at 100MPa and was completely degraded by 200MPa, which affected the texture of the muscle and led to a decrease in adhesiveness, gumminess and cohesiveness of the flesh. This produced a different texture to both raw and cooked cod. There were no other significant effects of pressure treatment at this level (on lipid stability and pH for example), suggesting that 200MPa and below may be the best treatment range for cod. However, lengthy treatment times also have a negative effect on fish quality (Dr. Patterson, *pers. comm.*) so it would be important to try and balance these effects.

The results from the SEM suggest that the HHP processing caused no damage to the external surface of *A. simplex* at 240MPa or 400MPa. Since the specimens were all apparently dead after the 400MPa treatment, the cause of death is likely to have been caused by internal damage to the organism. Unfortunately, since the sectioning techniques proved unsuitable for these animals, no further information could be gathered on this aspect.

#### 4.8 Chapter 4 Summary

A series of laboratory experiments were arranged to test the effects of basic environmental changes on the behaviour and mortality of *A. simplex* and *P. decipiens*.

The parameters tested were:

- Light (presence\absence)
- Partial desiccation
- Temperature (5°C - 30°C)
- Electrocutation
- Modified Atmosphere Packing (MAP)
- High Hydrostatic Pressure (HHP) treatment

Of these, only the HHP treatment had any effect on the mortality of the nematodes after 72 hours. Total mortality of *P. decipiens* (after 72 hours) was achieved using a minimum pressure of 240 MPa for three minutes. This was not achieved for *A. simplex*, but may reflect the lower pressures and shorter times used.

HHP had noticeable effects on the texture of the cod portions. While this was not quantified, it should be considered as a possible consequence of the treatment. Additionally, this treatment does not remove the nematodes from the cod muscle, which may still pose a problem for consumers. However, it may be an alternative to freezing the portions, as is currently done.

Further refinement of the HHP treatments could determine a minimum safe treatment time for *A. simplex* while confirming that determined for *P. decipiens*. Quantitative assessment of the cod portions would also determine whether this is a suitable process for the seafood industry.

## **Chapter 5: Further improvements: Suggestions from the literature**

### **5.1 Introduction**

The ideal method for removing the parasite problem would be to prevent the nematodes from entering the cod in the first place. Unfortunately, given the near ubiquity of *Anisakis simplex* and *Pseudoterranova decipiens* in the north Atlantic, this is unlikely to ever be achievable. Some alterations to current practice may be appropriate nonetheless, enabling fishermen to catch fish that naturally have fewer parasites.

### **5.2 The Importance of Fishing Grounds**

Several factors have been shown to influence the prevalence and intensity of anisakid infections in Atlantic cod and the distribution of parasites within the host tissues. Geographic location will influence which parasites are present and which hosts they will utilise. Platt (1975) found that Icelandic waters contained the most heavily infected cod of all stocks in the north-east Atlantic although he does not suggest reasons for this. Within that area however, cod from south-west Iceland had the lowest infestation rates, and he suggested this may be due to the influence of ‘clean’ cod migrating from Greenland to spawn.

As discussed in Chapter 2, Guðmundsson *et al.* (2006) studied the spatial and temporal distribution of ‘ringworm’ in fish caught by trawlers in Icelandic waters. Guðmundsson *et al.* (2006) may be a useful guide when sourcing cod to ensure that the vessels are fishing in areas where the nematode prevalence is relatively low. However, since long-liners were not studied and there is likely to be variation in nematode prevalence over time, other factors should also be considered.

Resident populations of pinnipeds and cetaceans have been shown to have some effect on the abundance of *A. simplex* and *P. decipiens*. Apsholm (1995) found a higher prevalence of *P. decipiens* in inshore, coastal regions (close to skerries and seal haul-out sites) compared to ‘offshore’ areas around the Norwegian coast. In Iceland, the common seal breeds around the entire coast, but congregates particularly in the north-

west (Dagbjartsson, 1973, Hauksson, 2006). The seals from the west and north-west coasts were also shown to have a higher prevalence of *P. decipiens* (Ólafsdóttir and Hauksson, 1998). Seasonal changes in the diet of the grey seals could also affect the transmission of parasites to different areas and should be considered (Ólafsdóttir and Hauksson, 1997). Avoiding such areas where seals are known to congregate may help to reduce the prevalence and abundance of parasitic nematodes.

Physical factors are also likely to affect the distribution and abundance of parasites in different regions. The eggs and early larval stages of *A. simplex* and *P. decipiens* are planktonic, and their distribution and the hosts they eventually infect will be affected by factors such as current regimes and stratification of the water column. For example, Klimpel and Rückert (2005) showed that stratification affected the prevalence and abundance of *Hysterothylacium aduncum* in North Sea fish. They found that stratification influenced the amount and type of food items available to predatory fish (particularly haddock and whiting) and therefore the prevalence and abundance of parasites in the fish. An examination of the oceanographic conditions around Iceland is beyond the scope of this study, but this aspect should not be overlooked when considering the total parasite burden of the cod stocks.

### **5.3 Alternative Detection Methods**

At present, candling is the only commercially viable method for detecting and removing nematodes from fish muscle, but is costly, time consuming and inefficient (Marcogliese *et al.*, 1996, McClelland, 2002). Several attempts have been made to design an alternative system that will improve the efficiency of detection in a commercial environment. Use of ultraviolet light (Pippy, 1970), x-rays and ultrasound for example have been suggested, but have proved ineffective on an industrial scale (Choudhury *et al.*, 2002, McClelland, 2002, Stormo *et al.*, 2004). Two promising potential alternatives are currently under investigation however, and will be discussed further.

### **5.3.1 Electromagnetic Detection**

Use of electromagnetism to detect nematodes in fish was first proposed by Choudhury and Bublitz (1994) and uses ‘superconducting interference quantum device magnetometry’ (SQUID) to detect changes in the electromagnetic conductivity between parasites and the fish flesh. Cod portions are placed between two electrodes in a saline bath and a current was passed through them to produce an electromagnetic ‘map’ of the fillet. Any nematodes in the flesh appear on the ‘map’ and can then be removed. This technique proved promising in laboratory conditions and has been developed further with a view to commercialising the system. Choudhury *et al.* (2002) report that this system will work effectively even when bloodlines or bones are present in the fillet, and regardless of the orientation of the nematode.

Electromagnetic detection is not without its problems however, and the authors do not indicate which parasite was used in trials, except that it was an anisakid. From the diagrams, it appeared to be quite a large nematode, and is most likely to have been *Pseudoterranova* sp. If this is the case, it throws doubt on whether *A. simplex* would be detectable by the system, as this species is much smaller and presumably would be harder to detect. Additionally, because this method requires the fillets to be immersed in saline, there could be quality control issues involved in its use. This system is also still in development, and it is unclear whether it is fast or effective enough to replace candling in the near future.

### **5.3.2 Imaging Spectroscopy**

Imaging spectroscopy relies on determining differences in the optical properties between fish muscle and parasitic nematodes. Recent work by Stormo *et al.* (2004) showed that both *A. simplex* and *P. decipiens* absorbed light of a different frequency to cod muscle, producing distinct absorption peaks. These data were used by Heia *et al.* (2007) to develop an automated system for visually identifying parasites within cod fillets.

Heia *et al.* (2007) carried out their study on cod portions that had been skinned, filleted and frozen, and contained encysted nematode larvae. The fillets contained blood spots and pieces of black membrane, and were in a similar condition to those used commercially. The fillets were then photographed, and the images analysed

automatically. Overall, the results appeared to be promising and all nematodes could be detected.

However, the detection rate was dependent on setting an appropriate threshold level in the image analysis software. Detection of all the parasites could therefore also result in 'false positives', where other imperfections in the fillets would be incorrectly highlighted as parasites. Additionally, the system was only able to detect nematodes embedded at up to 8mm depth. While this is approximately 2mm deeper than can be detected by standard candling procedures (McClelland, 2002), Hauksson (1989) found that approximately 5% of all 'roundworm' were found 1-3cm deep in the fillet. The imaging spectroscopy method would not improve the detection of deeply embedded parasites. However, it could be effective in detecting *A. simplex*, which may not be possible with electromagnetic detection.

Both systems discussed here are in their infancy and are not yet ready for commercial use. Both are driven by advancements in technology, and may simply need more time to improve the capabilities of the equipment. It is certainly possible that such systems will be able to provide an effective alternative to manual candling in the future.

#### **5.4 Chapter 5 Summary**

The ideal solution to the nematode problem would be to prevent the parasites from entering the cod muscle at all. Unfortunately, because of the high prevalence of these nematodes in the north Atlantic, it is unlikely that this can be achieved.

Nonetheless, it may be possible to reduce the problem by only fishing in areas that are known to have a low prevalence of parasites at any given time of year, using existing data. Avoiding areas where there are known to be high numbers of the definitive host species (e.g. whales or seals) could also help to reduce the parasite burden.

It may also be possible to modify the detection techniques used during processing. Candling (as used at present) is an inefficient and relatively ineffective method for detecting nematodes that are deep within the fillet, but is currently the best method available.

Two alternatives have recently been trialled and have produced promising results. The first of these involves electromagnetic detection of the nematodes, producing a 'map' of any nematodes in a portion of cod muscle. It was also able to detect nematodes in a range of orientations and in fillets of different thicknesses and quality. It is unclear which species this method is capable of detecting however, and it has not been trialled outside of the laboratory as yet.

The second method uses imaging spectroscopy to visually detect differences between the cod muscle and the nematodes within it. Again, this appeared promising and was able to detect all nematodes present to a maximum depth of 8mm. Candling can detect nematodes to 6mm however, so although an improvement, this method would not detect any parasites that are deeply embedded in the fillet.

## **Chapter 6: General Discussion**

The present study confirms that the nematodes present in the cod fillets used by the Macrae Food Group are two species of anisakid nematode: *Anisakis simplex* and *Pseudoterranova decipiens*. These are known to be pathogenic to humans and can cause ‘Anisakiasis’ if ingested alive (for example in raw or undercooked fish) (e.g. Sakanari and McKerrow, 1989, Audicana *et al.*, 2002, Akbar and Ghosh, 2005, Chai *et al.*, 2005).

The majority of both species were found in the cod viscera, although they were also present in the musculature. *Anisakis simplex* tended to cluster in the abdominal flaps and was less common in the fillets, whereas *P. decipiens* was equally common in each region. A third species, *Hysterothylacium aduncum*, was present only in the cod viscera, but is not pathogenic to humans and is unlikely to pose a significant risk to a consumer.

As discussed in Chapter 3, additional research into the development of molecular techniques would allow the precise subspecies of *P. decipiens* and *A. simplex* to be determined in Icelandic cod stocks. This would be of great benefit both scientifically, as a tool to understand the population and environmental preferences of each subspecies, and commercially, as a forensic tool for determining which nematodes result in most customer complaints.

There were no other significant trends in the distribution and abundance of the nematodes, for example between fishing vessels or fishing grounds. However, the present study was somewhat limited in its scope for analysing such trends and further work examining the Icelandic fishing fleet and seasonal and spatial trends would be beneficial.

The behavioural studies clearly showed that *Anisakis simplex* and *Pseudoterranova decipiens* are extremely difficult animals to kill or manipulate once they are encapsulated within cod muscle, and within the parameters that are useful to a commercial fish processor. While these trials were all on a very small scale and

would benefit from additional study, there was limited evidence that simple environmental changes could provide useful commercial benefits.

Of the treatments trialled, only HHP processing was effective in increasing the mortality of *A. simplex* or *P. decipiens*, although the mechanism by which it caused death is currently unclear. 100% apparent mortality of *P. decipiens* after 72 hours was achieved using a minimum treatment of 240MPa for three minutes, but this could not be achieved for *A. simplex*. Further study into determining the minimum acceptable treatment time and pressure would be required for *A. simplex*. Quantifying the effects on cod flesh would also be necessary to ensure that such treatments were commercially acceptable.

Molina-Garcia and Sanz (2002) report that a treatment at 200MPa for 10 minutes will kill all *A. simplex*, whereas Dong *et al.* (2003) report a pressure of 207MPa for 3 minutes is sufficient. The present study could not verify whether these treatments are effective. However, since 220MPa for 5 minutes was found to be ineffective in achieving 100% mortality of *A. simplex* in the present study, it seems unlikely that shorter treatments at lower pressures would be effective. All three studies were relatively small however, and should be viewed as preliminary investigations until larger trials can be carried out.

Although the microscopic sectioning was unsuccessful during the present study, it would be an area of research worth pursuing in order to determine the physical effects of pressure on the nematode parasites, and as a way of confirming death in the specimens. Sectioning may have been possible if the specimens had first been treated with a clearing agent (e.g. lactophenol) as this may have softened the cuticle enough to allow processing, and could form the basis of further work.

One conclusion that can be reached from the evidence in both the present study and previous literature is that there is a general lack of behavioural knowledge on *A. simplex* or *P. decipiens*. Ronald (1960, 1962, 1963) is the only known author to have systematically tested the effects of environmental changes on *P. decipiens*. Experiments on *A. simplex* have focussed on migration pattern in post-capture herring and mackerel (Smith and Wooten, 1975 and Smith, 1984), but there is a dearth of

information on the processes and stimuli that cause such behaviours. Further study based on the natural life cycle of the parasites (for example the effects of low pH and high temperatures) could be extremely beneficial in furthering scientific knowledge in this field.

Until such stimuli are better understood, it may still be possible to ensure that cod are caught from regions that are known to have a naturally low prevalence of nematodes, which could help to reduce the overall nematode burden. In Iceland, such areas are likely to be found off the south-west coast, around one of the major spawning grounds for cod with higher burdens around the major seal colonies in the west and north-west. Nonetheless, as more recent studies have shown (e.g. Guðmundsson *et al.*, 2006), there is no single region that will provide cod with a low parasite burden year-round, and long-term monitoring of fishery landings and movements of the cod stocks is necessary to fully understand fluctuations in the parasite load.

As technologies continue to improve, viable alternatives to manual candling may be developed to remove those nematodes that remain in the flesh. At present, imaging spectroscopy and electromagnetic detection of parasitic nematodes appear to be the most promising alternatives, although they are currently not suitable for use on a commercial scale.

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#### **Websites**

Crustastun website. Accessed on 13\03\2008  
<http://www.crustastun.com>

## **Appendix A: Adherence to Original Brief**

When this project was originally commissioned, a number of objectives and workplans were agreed, to guide the research into the parasitic nematodes appearing in MFG's cod fillets. These objectives have all been met. The workplans and completed research are detailed below.

### **WP1: Identification of the parasite**

- The parasites will be isolated, counted and preserved
- They will be identified according to their appearance under light and scanning electron microscopy. Candidate species are various sealworms, and *Anasakis simplex*.
- Histological sections of the worms will be prepared for light and transmission electron microscopy to confirm identification
- Comparison will be made between these fish, and others supplied more locally, or caught by the RV Aora in the Clyde Sea Area
- These identifications will be confirmed by comparison with reference data sets held by the Natural History Museum, London.

### ***Research Completed:***

All aspects of this work plan were completed, and are described in Chapter 2.

Cod from the Clyde Sea area were typically caught in low numbers and were too small to be comparable to the samples from Iceland.

### **WP2: Distribution of worms in the host tissues**

- The intensity of the worm burden will be determined, and the sizes of the worms measured (as this may affect their distribution).
- The frequency distribution of parasite numbers within the fish host will be measured
- The distribution of worms in the various organs and tissue systems at varying times *post mortem* and under different conditions will be determined by dissection
- Where necessary, this will be confirmed by histological sections

- Comparison will be made of prevalence and worm distribution in line caught and day-boat caught cod, to determine whether day boats are the highest source of worms
- Comparison will be made between these fish, and others supplied more locally, or caught by the RV Aora in the Clyde Sea Area.

***Research completed:***

All aspects of this work plan were completed and are described in Chapter 2, with the exception of the nematode lengths.

Cod from the Clyde Sea area were typically caught in low numbers and were too small to be comparable to the samples from Iceland.

**WP3: Current practice**

- Capture process and location, particularly in relation to depth and to seal populations
- Post-capture holding conditions (especially temperature)
- Slaughter methods
- Times between hauling, slaughtering, gutting and filleting
- A comparison of these timings between multi-day and single day boat trips
- The filleting process
- Procedures for detecting nematode worms
- Dispatch arrangements to Macrae Foods

By visiting the factory at Fraserborough, the further handling and examination procedures that occur there will be determined.

***Research completed:***

All aspects of this work plan were completed and are described in Chapter 1.

**WP4: Movement of nematodes post mortem**

- The total parasite load per fish and the intensity of infection
- The effect of fish size on parasite load
- The distribution of parasites in the various organs and tissues, particularly in the gut and in the muscle, at different times before and after gutting
- Changes in this distribution in relation to any variation in processing practices

***Research completed:***

All aspects of this work plan were completed and are described in Chapter 2.

**WP5: Migration rate of worms**

If the worm species found locally in fish are the same as those found in Icelandic fish, then using fish caught under controlled conditions by the RV Aora in the Clyde Sea Area the following parameters will be altered

- The time from hauling to slaughter
- The time from slaughter to gutting
- The method of gutting
- The time from gutting to filleting
- Holding temperatures
- Dwell times post-portioning
- Gas flushing in different gas mixes (CO<sub>2</sub>, N<sub>2</sub>, O<sub>2</sub>)

The effect of these changes on the numbers of worms appearing in fillets will also be measured. This will provide an estimate of the rate of migration of the worms into the muscle tissue from the gut, and will identify the main factors that affect it.

***Research completed:***

The cod from the Clyde Sea area were not comparable to the samples provided by MFG, and so the original workplan could not be completed. However, this was replaced by extensive laboratory-based experimental work examining the effects of various environmental factors on both the mortality and migration rates of the nematodes. This work is described in Chapter 3.

## **WP6: Novel methods for detection of nematode worms in cod fillets**

Working in conjunction with other consultants to Macrae Foods Ltd, the following methods for identification and isolation of nematode worms from cod fillets can be assessed and compared.

- Visual Inspection technology, based on digital or video imaging combined with image processing
- Thermal imaging using IR thermography

This might lead to the identification of a more sensitive and convenient method for screening for nematode worms than the currently used ‘candling’ methods.

### ***Research completed:***

All aspects of this work plan were completed by reviewing the existing scientific literature and are described in Chapter 4.

The development of a novel technology by the other consultants proved ineffective, and was therefore not examined in this study.

### **Additional Research Completed:**

In addition to the work detailed in the workplans, a significant amount of additional research was completed. This included:

- Laboratory-based manipulation of environmental conditions and their effects on both the mortality and motility of the parasitic nematodes.
- Examination of novel technologies to effect the mortality of the parasitic nematodes (electrocution, modified atmosphere packaging and high hydrostatic pressure treatment) in addition to their effects on migration and motility.
- Thorough review of existing scientific literature to identify environmental and industrial factors that may affect the parasite burden of individual cod.

Thorough examination of the literature to describe novel technologies which may prove useful in detecting parasitic nematodes in cod fillets in the future.