Centre for Environment Fisheries & Aquaculture Science

Cefas

Attendance report:

Training Workshop - Anisakis UV press method, Institute of Marine Research (Havforskiningsinstituttet) Bergen, Norway, 30-31 January 2023.

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1. Introduction

Anisakis is a genus of parasitic nematodes that can cause Anisakiasis, a gastrointestinal disease in humans who consume raw or undercooked seafood. In recent years, there has been a significant increase in reported cases of Anisakiasis worldwide (Audicana et al., 2008), highlighting the importance of understanding the biology, epidemiology, and clinical manifestation of this parasitic infection.

Anisakis is a nematode with a complex life cycle that involves small crustaceans to fish (and/or cephalopods) as intermediate hosts. The adult worms typically live in the stomachs of marine mammals such as seals, sea lions, and whales, where they reproduce, with eggs shed in the host's faeces. The eggs hatch in seawater and develop into larvae, which are known as L1 larvae. The L1 larvae are ingested by small crustaceans such as krill or copepods, which serve as intermediate hosts. Inside the crustaceans, the L1 larvae moult into L2 larvae, which are then eaten by fish (or squid). The larvae penetrate the intestinal wall of the fish (or squid) and migrate to the muscle tissue, where they form cysts. This stage of the parasite – L3 larvae - is also capable of surviving in the digestive tract of other mammals, including humans, although it is incapable of reproducing there. Consuming raw or undercooked seafood containing the infected L3 larvae lead humans to become accidental hosts. This may result in gastrointestinal pain and upsets, amongst other possible symptoms. The worms (dead or alive) can also act as allergens (EFSA, 2010). The nematode species causing human infection in the NE Atlantic region are *A. simplex* and *Pseudoterranova decipiens* (Petrie et al., 2010). The final hosts are cetaceans (*A. simplex*) and pinnipeds (*P. decipiens*). The final host affects the distribution of the parasite in the marine environment and the range of intermediate hosts which become infected (EFSA, 2010). They are found in many commercially significant species harvested from the NE Atlantic and Mediterranean regions including demersal (e.g. gadoids) and pelagic species (e.g. mackerel and herring).

The L3 larvae of these nematode species are most frequently found in fish viscera rather than the musculature but can migrate from the former to the latter in ungutted fish *post-mortem* (EFSA, 2010). Both species tend to form characteristic 'encysted coils' within their fish hosts.

A. simplex averages 20mm in total length and is easy to see in the viscera, but much less obvious when embedded in fish muscle. *P. decipiens* is a slightly larger and more darkly coloured worm, so is more obvious in muscle tissue. Their differing life cycles affect their occurrence in fishery products.

Measures to control zoonosis include inactivation through freezing or cooking and screening during processing, where individual worms can be removed, or heavily infected material rejected. Gutting and icing as soon as possible after capture can prevent post-mortem migration from viscera to flesh. Products containing viable or visible larvae are unlikely to be well received by consumers, further reinforcing controls applied by the seafood supply chain.

Legislative controls in the UK are specified in retained European Regulation (EC) No. 853/2004, as amended and subsequently copied in part into UK law through Statutory Instrument 2019 No. 1247. Fishery products intended for consumption raw (or not heated to 60°C or above for at least one minute) must be frozen at -20°C for at least 24 hours before being placed on the market. Other products intended for cooking must have been subject to visual examination and must not be marketed for human consumption if they are contaminated with parasites. This definition is somewhat ambiguous but has been further detailed in European Commission Regulation (EC) No 2074/2005. This defines the term "visual inspection" as a "non-destructive examination of fish or fishery products with or without optical means of magnifying and under good light conditions for human vision, including, if necessary, candling". Candling is defined as "in respect of flat fish or fish fillets, holding up fish to a light in a darkened room to detect parasites". This must be done continuously during manual evisceration and washing by qualified persons; in the case of machine processing, a representative number of samples should be inspected.

These controls are implemented by food business operators, under the supervision of Local Authorities in the UK. Methods employed by the seafood industry to ensure their products meet the hygiene requirements are either visual examination or candling. Visual examination is a simple inspection of the product in well-lit conditions. Candling usually involves examining fish fillets over a light box, whereby a diffuse white light is shone through the specimen from underneath. No official testing is undertaken in the UK in support of the legislative requirements.

There are two validated methods for which ISO standards have recently been developed:

- artificial digestion (BSI EN ISO 23036-2:2021 Microbiology of the food chain – Methods for the detection of Anisakidae L3 larvae in fish and fishery products – Part 2: Artificial digestion method) and
- UV-press (BSI EN ISO 23036-1:2021 Microbiology of the food chain – Methods for the detection of Anisakidae L3 larvae in fish and fishery products – Part 1: UV- press method).

The artificial digestion method uses enzymatic degradation of the sample in a fluid composed of pepsin and hydrochloric acid followed by filtration and washing. *Anisakis* larvae are highly resistant to digestion and can be enumerated and assessed for viability. The UV press method involves pressing the material to about 2mm thickness, freezing it to kill the larvae, and then viewing the sample under UV light. *Anisakis* nematodes fluoresce after death under UV light and are counted by the analyst. It is possible to detect almost all larvae within a sample using either of these methods, although the viability of the larvae can only be assessed by the digestion method as the UV-press method kills them. The ISO standards indicate the accuracy, sensitivity, and specificity of the digestion method are 98%, 96% and 100%, and for the UV press method, they are all 100%. Both express results as a number of larvae per unit weight of the sample.

The UK National Reference Laboratory (NRL) for Anisakis at Cefas was tasked by the Food Standards Agency to progress the accreditation of its UV-press method to ISO17025 standard to meet the statutory requirement for NRLs to only use accredited methods. Whilst the method has been in place at Cefas for many years, only very limited testing has been undertaken by the laboratory and accreditation had not been a requirement. The new requirement for accreditation meant that the method had to be formally characterised by Cefas and the various steps of the process defined and specified in such a way that it met all elements of a quality system. To Cefas knowledge, there are no official laboratories in Europe which are designated for Anisakis testing and accredited to ISO17025 standard for this testing by either the digestion or the UV press method. It has therefore not been possible for the NRL to seek advice from an accredited laboratory on the use of the method. Instead, the NRL contacted a Norwegian laboratory known for their extensive use of the method and their international expertise in this field to seek this advice.

This report aims to provide a comprehensive overview of the UV press method as witnessed during a training workshop on the *Anisakis* UV press method organised by The Institute of Marine Research (IMR) in Bergen (Norway) in January 2023. The IMR hosts the National Institute of Nutrition and Seafood Research Centre with its Seafood Hazard group led by Senior Scientist Arne Levsen who organised the workshop. The workshop was delivered by Lucilla Giulietti (Post Doctoral Research) and Paolo Cipriani (Research) at the IMR in Nordnesboder 4, Bergen.

2. Objective

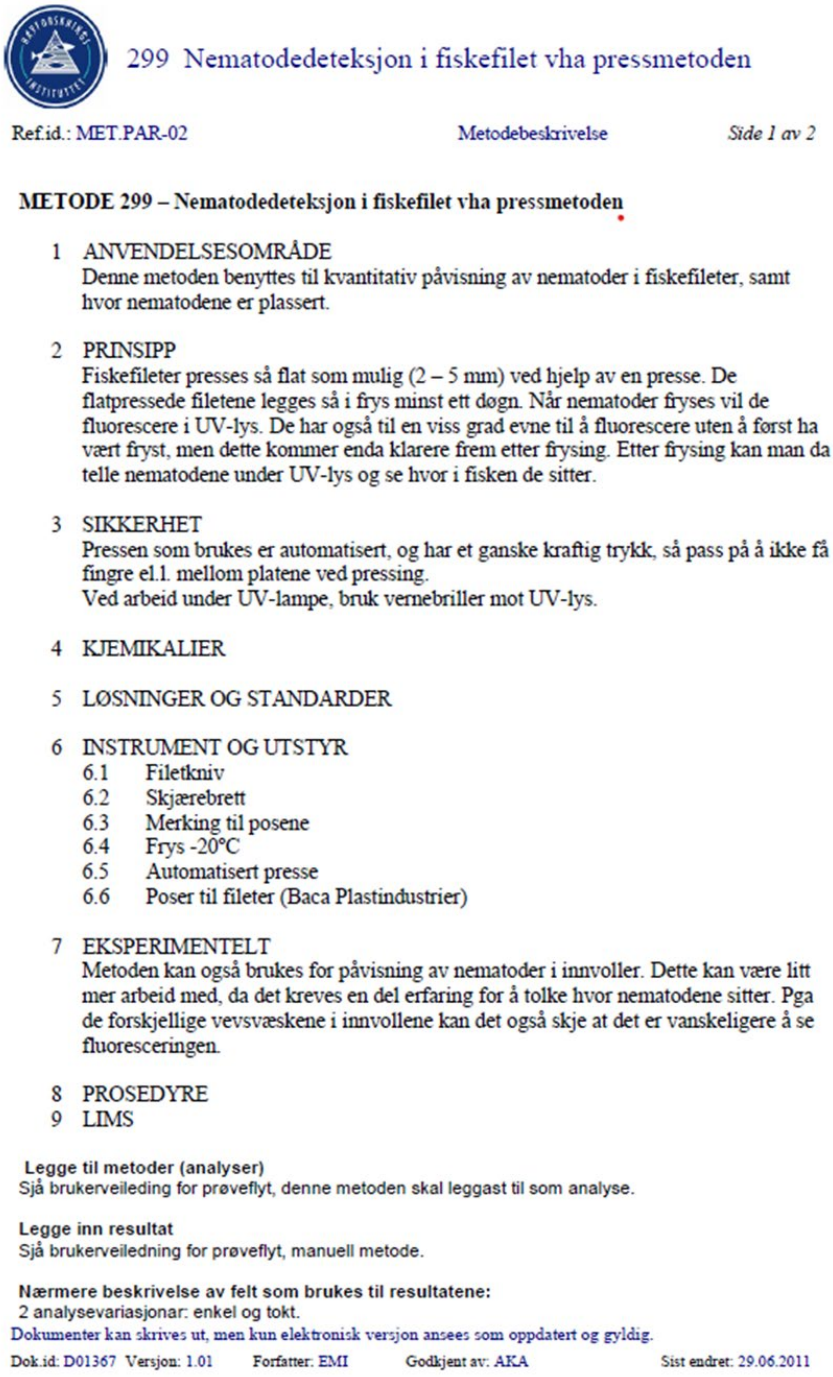
The training workshop had the objective to familiarise the participants with the existing UV press method, following the ISO 23036-1 Microbiology of the food chain – Methods for the detection of Anisakidae L3 larvae in fish and fishery products – Part 1: UV-press method.

The workshop also intended to help recognise and identify the *Anisakis* species through morphological ID keys, as well as to acquaint the participants with the equipment used during the UV process. Participants were asked to focus on the correct technique and methodology for this laboratory procedure and identify critical control points (CCPs) for developing guidelines to achieve national accreditation in both organisations (Cefas and IMR). CCPs were identified and discussed at the end of the training session.

IMR uses the UV press method on a large volume of samples each year. Observing the method in use in a working laboratory and the logistics involved was also helpful to the NRL's understanding of the method and the elements of the process which need to be specifically covered in quality documentation.

3. Activities of the training

The training was divided into two days: the first session consisted of a presentation by Lucilla Giulietti (Post Doc) about the IMR and the Seafood Hazard group research works along with the protocol used in IMR for this specific procedure (see Figure 1). This was followed by the first practice session in the lab. On the second day, we analysed the results obtained under UV light, then discussed the identified CCPs.



299 Nematodedeteksjon i fiskefilet vha pressmetoden

Ref.id.: MET.PAR-02 Metodebeskrivelse *Side 1 av 2*

METODE 299 – Nematodedeteksjon i fiskefilet vha pressmetoden

1 ANVENDELSESOMRÅDE
Denne metoden benyttes til kvantitativ påvisning av nematoder i fiskefileter, samt hvor nematodene er plassert.

2 PRINSIPP
Fiskefileter presses så flat som mulig (2 – 5 mm) ved hjelp av en presse. De flatpressede filetene legges så i frys minst ett døgn. Når nematoder fryses vil de fluorescere i UV-lys. De har også til en viss grad evne til å fluorescere uten å først ha vært fryst, men dette kommer enda klarere frem etter frysing. Etter frysing kan man da telle nematodene under UV-lys og se hvor i fisken de sitter.

3 SIKKERHET
Pressen som brukes er automatisert, og har et ganske kraftig trykk, så pass på å ikke få fingre el.l. mellom platene ved pressing.
Ved arbeid under UV-lampe, bruk vernebriller mot UV-lys.

4 KJEMIKALIER

5 LØSNINGER OG STANDARDER

6 INSTRUMENT OG UTSTYR
6.1 Filetkniv
6.2 Skjærebrett
6.3 Merking til posene
6.4 Frys -20°C
6.5 Automatisert presse
6.6 Poser til fileter (Baca Plastindustrier)

7 EKSPERIMENTELT
Metoden kan også brukes for påvisning av nematoder i innvoller. Dette kan være litt mer arbeid med, da det kreves en del erfaring for å tolke hvor nematodene sitter. Pga de forskjellige vevsvæskene i innvollene kan det også skje at det er vanskeligere å se fluoresceringen.

8 PROSEDYRE
9 LIMS

Legge til metoder (analyser)
Sjå brukerveileding for prøveflyt, denne metoden skal leggast til som analyse.

Legge inn resultat
Sjå brukerveiledning for prøveflyt, manuell metode.

Nærmere beskrivelse av felt som brukes til resultatene:
2 analysevariasjonar: enkel og tokt.

Dokumenter kan skrives ut, men kun elektronisk versjon ansees som oppdatert og gyldig.

Dok.id: D01367 Versjon: 1.01 Forfatter: EMI Godkjent av: AKA Sist endret: 29.06.2011

Figure 1. IMR Protocol for UV press method

4. *Anisakis* UV press procedure

During the first day, the IMR team collected data in regards the fish origin (vessel name and coordinates of fishing catchment area), then fish (Herrings, *Clupea harengus*) were enumerated (100 fish in total), and the length and weight collected through an electronic fish measuring board and saved on a spreadsheet.

Figure 2. Fish sample and the electronic fish measuring board.

Then the IMR team prepared 3 different sizes of clear plastic bags (as described in ISO 23036-1), and labelled each of them with a unique identifier and an abbreviation to identify which organ and or which side of fillets will be placed inside (for i.e., V1 for viscera of fish n.1, L1 left fillets of fish n.1, R1 right fillet of fish n.1 and so on). However, IMR does not use any serial number based on different fish species and does not use a unique serial number for each entry for the *Anisakis* UV press method (see CCPs).

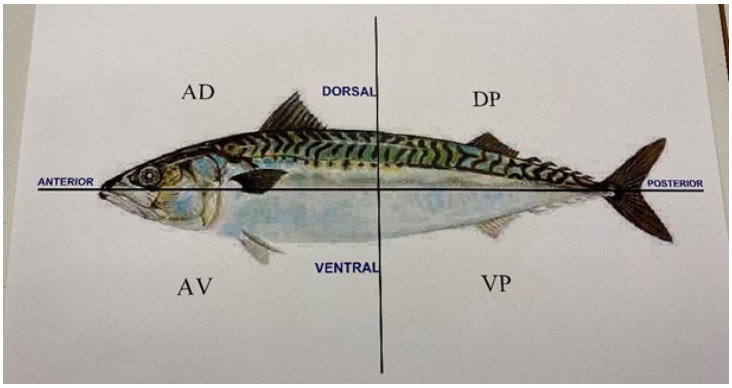


Figure 3. Key to labelling of fish fillets used by IMR

Figure 4. Removal of viscera and viscera and fillets in 2 different plastic bags.

Once the clear plastic bags were labelled, the IMR team filleted and removed the viscera (which were individually placed in a labelled bag i.e. V1 to be checked separately), as well as each side of the fish fillets (left and right). It is good practice to separate viscera from the fillets, as they would jeopardise the results due to the complex texture of the organs). When all the fish fillets were ready and divided into different plastic bags, the IMR team proceed to use the automatic pressure machine held at the lab.

The automatic press machine held at the IMR has 2 metallic trays approximately 5 cm wide on each side that compress, the fillet up to 1 to 2 mm. Once the fillets are all compressed and completely flat, the bags are placed in different trays on a freezer at -20°C overnight. Two observations on the process used by IMR which would need to be addressed prior to accreditation (see CCP list):

- There is no gauge valve on the IMR automated press and therefore the pressure applied to the fillets cannot be measured and confirmed to meet that defined by the ISO standard
- The team does not measure the final thickness of every single pressured plastic bag to make sure that post-compression, the fillet is within the 1-2mm range specified by the ISO standard. A quick check is performed by eye without the use of a micrometre.

Figure 5. Automatic press machine in use at IMR

The following day, the IMR team removed the trays from the freezer to allow the fillets to defrost before checking them under the UV light. When the fillets were ready, the IMR team proceeded (with special UV glasses and a pair of gloves for health and safety measure) to inspect and check the fillets and the viscera bags under the UV light box. This specific UV light box had a UV bulb not just on the bottom but also on the top of the screen for a better reading of the sample. See attached pictures showing how compressed fish fillets appear under the UV lights and the presence of *Anisakis* parasites in some of them. Observations on the process followed by IMR and which would need to be addressed prior to accreditation:

- The time during which fillets are defrosted is not recorded or monitored (roughly 1 hour or so);
- No thermometer is used to check the temperature of the fillets or the temperature of the room;
- The UV light system is handmade.

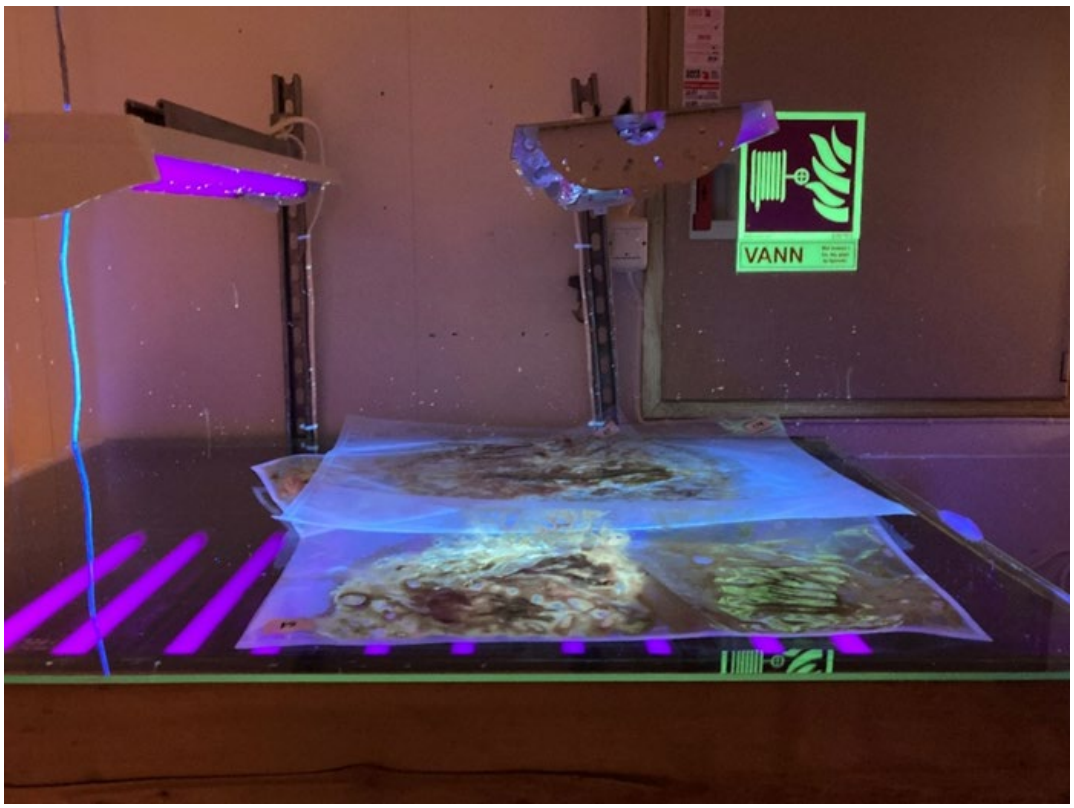


Figure 6. Handmade UV light box at IMR. Note the presence of UV bulb also on the top and not just on the bottom.

During the examination of the fillet, when parasites were found, the IMR team would highlight them by drawing a circle around the *Anisakis* with a black marker. This procedure is applied for any parasites spotted, followed by counting the total number of nematodes for fillets. The IMR technician would write the sum of the total parasites on a clear area of the plastic bag. Once the first reader (IMR technician) finishes analysing all the fillets, another technician or second reader will read and check again all the fish fillets. This procedure allows a double check on the reading, and where any parasites were previously missed, they are added to the total count. In case the IMR technicians are not confident about the identification (because the sample is “dirty”) or they believe it does not look like *Anisakis* (IMR technicians do not rely solely on the visual check under the UV light but also on the texture of the parasite itself), they would take the parasite out of the plastic bag and analyse it by using the morphological key identification under the microscope. Once all the fillets are examined (twice), the second reader will update a spreadsheet with all the data collected.

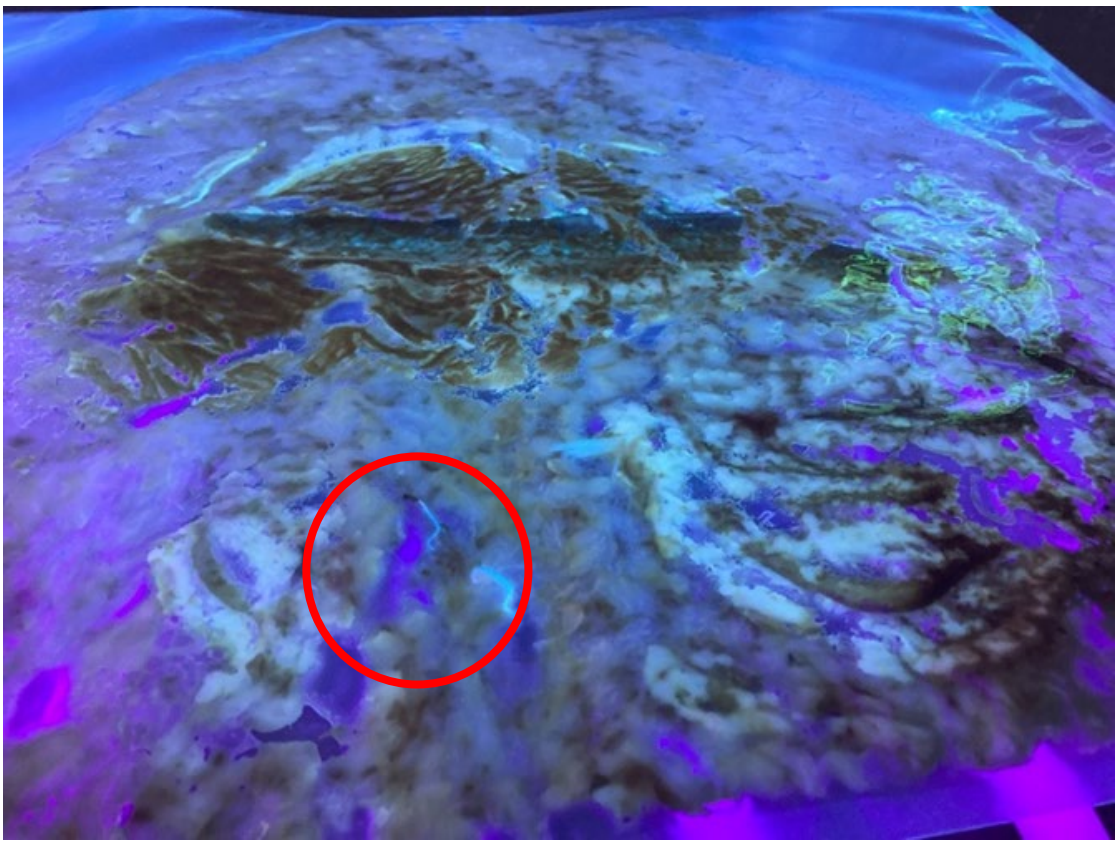


Figure 7. Highlight of 2 *Anisakis* nematodes in fish fillet.

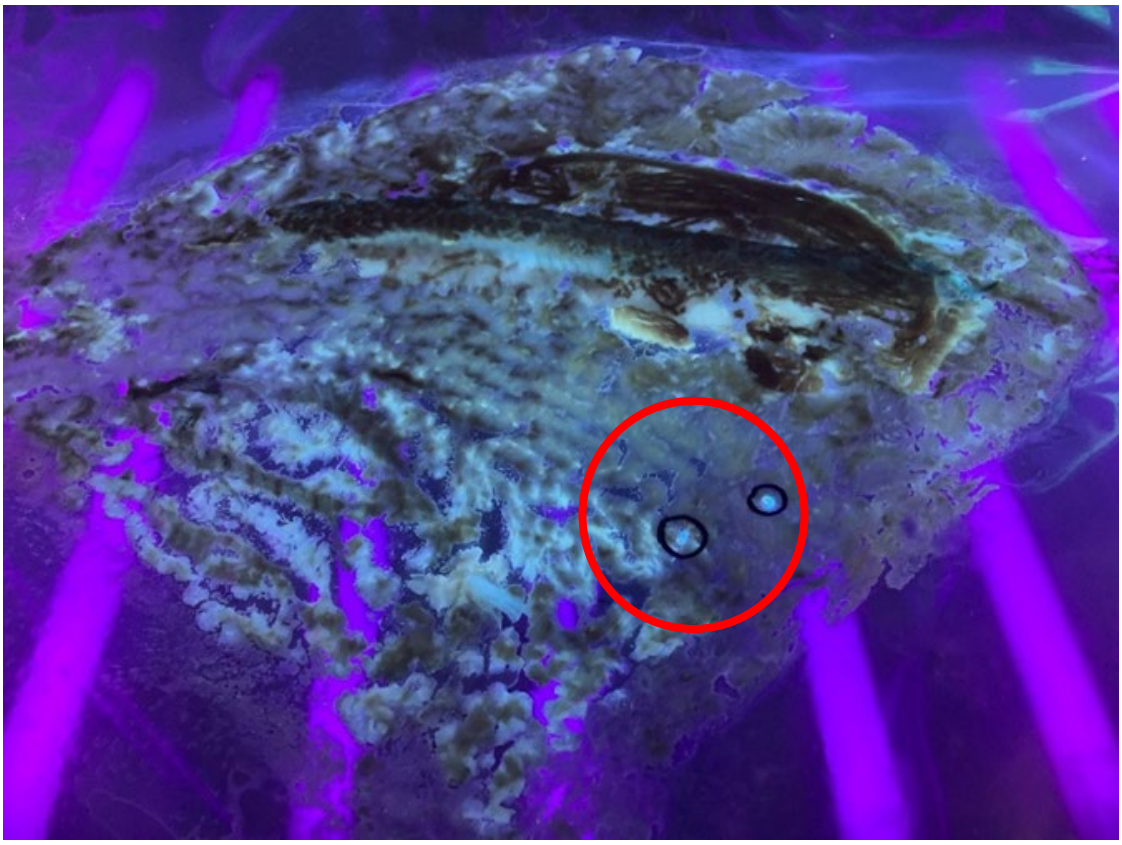


Figure 8. Highlight of 2 *Anisakis* nematodes in fish fillet.

Figure 9. Highlight of 2 *Anisakis* nematodes in fish fillet.

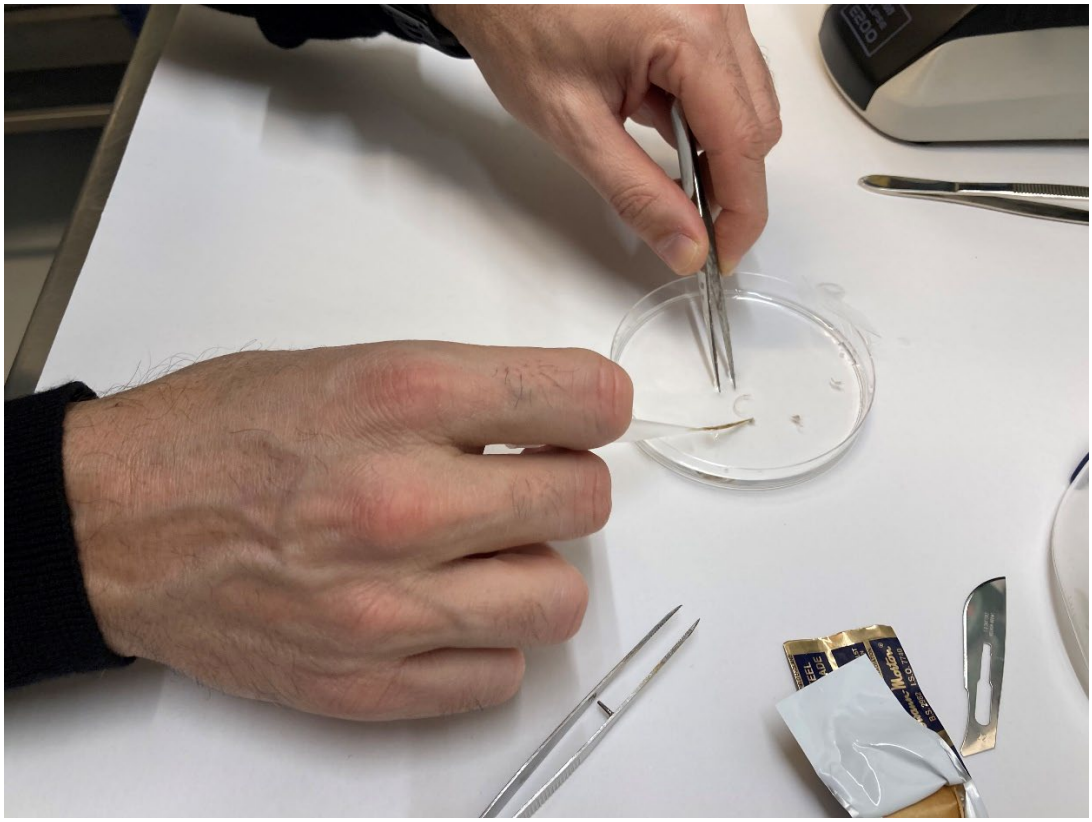


Figure 10. IMR technician isolating a nematode for further identification on the microscope.

5. Critical Control Points (CCPs)

Critical Control Points or CCPs is a term used in the Hazard Analysis and Critical Control Points (HACCP), a food safety management system that is used to identify and control potential hazards throughout the food production process. In this instance, the CCPs have been used to identify the steps in the UV press method, where a control measure can be applied. The correction of the CCPs identified will help Cefas refine its standard operating procedure (SOP) for the *Anisakis* UV Press and seek accreditation.

During the training some issues were identified at a number of CCPs:

- When samples arrive at the lab, the IMR do not have a procedure for labelling individual samples with a unique identification using a serial number or an alphanumeric number.
- The automatic press machine does not have a pressure gauge and therefore it is not possible to measure and control the pressure applied to each sample.
- The ISO standard does not specify a definition of “room temperature”, therefore the IMR team just leave the frozen fillets for 1-2 hours to defrost and do not check the temperature at the core of the product and assume the fillets will be suitable for pressing.
- The UV box used by the IMR team it is a handmade system, therefore it is not possible to apply the laboratory health and safety measures required by UK legislation.

The first CCP can be easily fixed/corrected by ensuring the lab introduces a booking in system were for each fish species analysed a unique reference code is used. This will avoid any possible confusion, help traceability and build up a better database for epidemiological purposes.

For the second CCP identified, the lack of a pressure gauge in the pressure machine can be easily rectified. However, as a standard laboratory procedure the equipment should be calibrated first and regularly monitored for compliance with the correct value. Calibration and ongoing monitoring must therefore be implemented.

On the third CCP, measures should be included in the procedure to check the room temperature and the temperature of the fillet.

The last CCP regarding the UV light raised some health and safety concerns as well as issues with the accredited process itself. For the development of an internal SOP at Cefas the document would require evidence of the correct functioning of the equipment and a correct calibration of the tools.

6. Conclusion

The UV press method represent an easy and straightforward process to analyse and identify the *Anisakis* in fish fillets. This method does not require highly specialised technicians and it can be delivered after proper training. Through the UV press, is it possible to identify the parasite location in each fish fillet (anterior dorsal/ventral – posterior dorsal/ventral) and also to isolate the nematode for a subsequential identification on a species level.

The IMR use the UV press method in their mainland laboratory and on one of the vessels of their fleet. The UV press allows the IMR researchers to build an epidemiological database for *Anisakis*. By screening and providing sufficient presence / absence data for *Anisakis* in specific fishing grounds in the North Sea, they hope to be able to provide evidence that might limit the requirement for freezing wild fish catches.

This workshop provided hands on demonstration of the UV press method and equipment in use in a laboratory with high sample throughput, something which UK official laboratories do not have experience of. Our understanding of accreditation enabled Cefas to identify some key issues at CCPs that would need addressing to meet the ISO17025 standards at both Cefas and IMR. The IMR is currently focusing on training more technicians in this procedure since they already have in place most of the correct equipment and routinely use this technique in their laboratory. IMR will pursue accreditation but they have identified that it is likely to take them a minimum of 2 years before they can apply for accreditation for the UV press method.

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We are the government's marine and freshwater science experts. We help keep our seas, oceans and rivers healthy and productive and our seafood safe and sustainable by providing data and advice to the UK Government and our overseas partners. We are passionate about what we do because our work helps tackle the serious global problems of climate change, marine litter, over-fishing and pollution in support of the UK's commitments to a better future (for example the UN Sustainable Development Goals and Defra's 25 year Environment Plan).

We work in partnership with our colleagues in Defra and across UK government, and with international governments, business, maritime and fishing industry, non-governmental organisations, research institutes, universities, civil society and schools to collate and share knowledge. Together we can understand and value our seas to secure a sustainable blue future for us all, and help create a greater place for living.

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