WORKSHOP ON THE MANUAL FOR GENETIC SAMPLING FROM FISHERIES PRODUCTS IN THE NAFO AREA (WKGENMAN)

VOLUME 3 | ISSUE 1

ICES SCIENTIFIC REPORTS

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ISSN number: 2618-1371

ICES Scientific Reports

Volume 3 | Issue 1

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Recommended format for purpose of citation:

ICES. 2021. Workshop on the manual for genetic sampling from fisheries products in the NAFO area (WKGenMan).
ICES Scientific Reports. 3:1. 21 pp. https://doi.org/10.17895/ices.pub.7692

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Contents

i Executive summary

The Workshop on the manual for genetic sampling from fisheries products in the NAFO area (WKGenMan) worked to respond to a special request for advice from the European Commission on the International Manual of Procedures for genetic sampling (IMP), an EU proposal to the Standing Committee of International Control (STACTIC) of the Northwest Atlantic Fisheries Organisation (NAFO) to guide the collection of samples from fisheries products for genetic analysis that can result in unequivocal species identification. In this report the workshop reviews the adequacy of the IMP for guiding the collection, preservation, and analysis of genetic material. During the review a series of recommendations to clarify/improve the manual were highlighted. In particular, it was emphasized that the protocol should be tuned after preforming a pilot study in real conditions and that if samples are correctly collected and stored, they could last in good condition for months, which is much longer than the original five days stated in the protocol. In general, it was appreciated the importance of the manual to strengthen the ability to control for compliance with species labelling, contribute to a stronger fisheries control and enforcement capacity and to a more efficient fight against Illegal, Unreported and Unregulated fishing.

ii Expert group information

Expert group name	Workshop on the manual for genetic sampling from fisheries products in the NAFO area (WKGenMan)
Expert group cycle	Annual
Year cycle started	2020
Reporting year in cycle	1/1
Chair(s)	Naiara Rodriguez-Ezpeleta, Spain
	Jann Martinsohn, Italy
Meeting venue(s) and dates	12 January 2021, by correspondence (8 participants)

1 Terms of reference a) Review the adequacy of the approach proposed in IMP for collection of genetic material for sampling of fish on board vessels for species identification

Summary of provided information

The International Manual of Procedures for genetic sampling (IMP) was developed as a guide for the collection of samples of biological material from fisheries products for species identification based on genetic analysis in the NAFO Regulatory Area by inspectors from NAFO contracting parties.

The intended use of the samples ranges from mere information to the provision of evidence in a control and enforcement context. The latter demands the establishment of the Chain of Custody, as an inherent component underpinning the sample collection process. The chain of custody is described in the introduction and objectives of the IMP.

Regarding the sample collection, the manual presents a procedure based on a formula developed by Cochran [1]. The description of the kit for the collection of samples and a form for sample collection to be filled by inspectors are also provided. In addition, the manual presents three case studies for the estimation of the sample dimension for American Plaice, Cod and Thorny Skate and two statistical tables to be used for the calculation of sample dimensions.

Observations

The proposal to apply genetic approaches to control compliance with species labelling is clearly in line with Article 13 of the Common Fisheries Policy Control Regulation [2].

This is not the first attempt in providing a guide for the collection of samples from fisheries products for genetic analysis, but it is the first time that DNA-techniques will be used at a large scale in European waters (and beyond) to monitor for compliance with species labelling rules. In addition to the Portuguese Manual of procedures, which was the starting point for the elaboration of the IMP, another relevant example is the test project carried out by the Danish AgriFish Agency in collaboration with the Danish academic institution DTU Aqua. The test explored the feasibility for fishery inspectors to undertake sampling for DNA-analysis; in doing so, they produced a simple guide on the use of DNA for fisheries control and developed a control toolbox and pre-formatted report delivery note, which enables fishery inspectors to carry out tissue sampling *in situ* [3]. With respect to (genetic) sampling strategies the Office of Law Enforcement and Marine Forensics Laboratory of the US National Oceanic and Atmospheric Administration (NOAA) supported the legal investigation into a large-scale false labelling scheme of catfish imported in the United States of America and intentionally mislabelled as higher value fish species [3]. This required the sampling of containers full of frozen and filleted fish, based on elaborate guidelines.

The IMP provides an extensive description of the methodology to be used to estimate the number of samples to collect and to select the boxes to be sampled; however, only the following sentence

has been devoted to the procedure of taking samples from a box (paragraph "Sampling", page 8): "From each box, a fishery product is randomly chosen for sampling." It would be useful to include in the IMP guidelines on how to carry out the choice of the fish or product to sample from a box. It is important to recommend a robust, standardized and well-documented sampling approach at this stage of the procedure in the event of a need to take a case to court. Importantly it has also to be considered that the fish (product) is normally frozen, which further complicates the sampling. Moreover, it is not clear from the text, whether the protocol refers exclusively to the sampling of landed fish (at the port) or sampling on board of a vessel. If the latter, it should be ensured that systematic sampling is a feasible option, in light of the often difficult working conditions on board of a vessel.

At page 9 of the manual, a simplified formula is introduced that can be used with the assumption that "the identities of the species being investigated are distributed normally or nearly so". We observe that there is no further explanation relating to this assumption in the IMP nor references to external documentation allowing the inspectors to infer when and for which species this important assumption can be considered valid. This might also be an important consideration in the context of legal investigations and court cases.

The proposed sampling design is based on previous knowledge of the probability of detecting mislabelled species in the target geographical area. The case studies presented in the IMP use catch data from previous years to define this probability. We observe that the degree of reliability of those data (presumably provided by fishers) in relation to the correct identification of the involved species is not discussed.

Due to the high number of samples that inspectors will have to collect on average, the size of the kit used for the collection of the samples constitutes a relevant aspect to be considered when inspectors plan their sampling trips.

Conclusions and recommendations

- As clearly stated in the IMP, we support and emphasize that an appropriate training for inspectors in charge of the sampling is essential to the successful implementation of methodology described in the manual. The European Fisheries Control Agency (EFCA) is an appropriate European body to provide such training;

- Prior examples such as genetic sampling in Denmark or of the false labelling of catfish in the US should be used as informative case studies. We recommend that contacts should be established between respective involved authorities to mutually profit from relevant experience;

- The IMP mentions also the fundamental need for a pilot period of application of the current version of the IMP. We agree and recommend foreseeing the proposed pilot test in order to verify the technical suitability of the approach described in the IMP and to estimate the probability values required the statistical approach underpinning the sampling method [1], also in light of the observations depicted above. Ideally, any such pilot study should be accompanied by experts in the field, such as staff of the Office of Law Enforcement of the US National Oceanic and Atmospheric Administration (NOAA);

- In the context of the simplified sampling formula, we suggest including in the manual an explanation relating to the assumption that "the identities of the species being investigated are distributed normally or nearly so" and preparing some case studies for the inspectors to exemplify for which species and/or in which cases the assumption is applicable;

- We suggest that in the manual the degree of reliability of species data (presumably provided by fishers/the fleet) in relation to the correct identification of the involved species will be further discussed. We suggest adding to the IMP some guidelines about how to carry out the choice of the fish or product to sample from a box. We also recommend including a picture in the paragraph "Collection" (page 12) to illustrate the procedure to follow when selecting the boxes to be sampled. The description itself is rather intricate (from IMP, page 12): "*Assuming a rectangular transversal and longitudinal section, this requires the notional division of the lot in 27 sub-lots (nine for the shallower, middle, and deeper layer each, corresponding to a central sub-lot adjoined by 4 mid-edge and 4 corner sub-lots) and that fish is taken in similar numbers from each sub-lot (which may correspond to one or more fish boxes). Whenever sample size is lower than 27, inspectors should pick one of the sub-lots randomly and sample the opposite sub-lot as well as the intermediate one for the minimum sampling size of 3 boxes and repeat this procedure for sampling sizes between 3 and 27 until reaching the intended sampling size.*".

Sources and References

[1] Cochran, W. G. 1963. Sampling techniques, 2nd Ed. New York, USA: John Wiley and Sons, Inc.

[2] COUNCIL REGULATION (EC) No 1224/2009 of 20 November 2009 establishing a Community control system for ensuring compliance with the rules of the common fisheries policy.

[3] Martinsohn, J. T. et al. DNA-analysis to monitor fisheries and aquaculture: Too costly? Fish Fish. 20, 391–401 (2019).

[4] DGRM. 2015. Directorate-General for Natural Resources, Security and Maritime Services (DGRM) of the Ministry of Agriculture and the Sea. Recolha de amostras de produtos da pesca para testes de adn manual de procedimentos – Portuguese Manual of Procedures (PMP). 11 pp.

2 Term of reference b) Evaluate feasibility and recommend a method for sample preservation that will be effective at maintaining sample integrity in the NAFO Regulatory Area fisheries, where at least 7 days are needed from sample collection to delivery at a laboratory facility

Summary of provided information

The protocol recommends collecting muscle or fin tissue samples and suggests that samples to be collected should be between 2.0 to 4.5 cm^3 (either 2x2x0.5cm or 3x3x0.5cm) and that they should be divided into three equal size fragments that are stored in three independent vials containing ethanol 96% or RNA*later*™. The protocol clearly states that the samples should be fully immersed in the preservation liquid (ethanol or RNA*later*™). The protocol states that the sample vials should be placed in a thermal box for optimal storage during transport. Then the protocol specifically states that the "maximum period of delivery of the sample in the laboratory is 5 (five) working days after sample collection".

Observations

As result of the on-going EFCA consultation, DGMARE (D4), an implementation issue was identified in relation to the number of days recommended in the protocol (maximum of 5) for transferring genetic samples from collection sites to delivery at the laboratory. According to EFCA, at least a maximum period of 7 days should be sought. We advocate that if the sample is correctly preserved, it could be stored for more than 5 days (even several years) without losing integrity and being perfectly valid for genetic analyses. What is crucial for ensuring sample integrity is 1) the time between the death of the fish and the conditions at which the dead fish is stored; 2) the preservation materials and method of the collected sample [1].

Sampling: Samples should be collected as soon as possible after landing catch on deck and, if not possible, catches should be preserved at the lowest available temperature (or frozen) and protected from light. These conditions following death minimise impacts of DNA degradation (fragmentation into smaller pieces). The DNA degradation process is slowed at lower temperatures and stopped if fish is frozen.

Sample preservation: Once collected, samples should be preserved in conditions that ensure its integrity over time, aiming at storing the sample for as long as several years. Both methods suggested in the protocol, ethanol 96% and RNA*later*™ are good preservatives for samples collected for genetic analyses. In both cases, it is crucial that the ratio sample: preservative is at least of 1:5 and that tissue sample should not be larger than 0.5cm^3, which implies that the tissue sample from which the three subsamples should be collected should not be larger than 1.5 cm^3.[1] Since sample tissues can contain water, for long storage (a few months), it is advised that ethanol is

[1] This point was added as a response to the reviewers in Annex 3. Please see Annex 3 & 4 for more details.

replaced with equal volume after 24h to ensure sufficient concentration. Also, even though room temperature is fine for a few hours or days, for longer storage, ethanol preserved samples should be maintained at -20C. Moreover, ethanol to be used should be analytical/molecular grade, as other grades (100% or denatured) may contain sub products that degrade the DNA. The advantage of ethanol over RNA later is that it is less expensive; however, RNA*later*™ allows easier sample transportation (as it is not flammable and thus does not require special transportation permits).

Conclusions and recommendations

- We recommend that the protocol removes the reference to the maximum number of days from sample collection laboratory delivery since, if samples are correctly handled and preserved, there should be no time limit. Instead, the protocol should clearly specify that:
    - the sample should be collected as soon as possible after the fish has been caught or the fish to be sampled should immediately be frozen for subsequent sample collection;
    - ethanol of equal volume should be replaced after 24h for long term storage of samples at -20°C;
    - ethanol to be used should be analytical or molecular grade.

References

[1] Rodriguez-Ezpeleta, N., Mendibil, I., Álvarez, P. and Cotano, U., 2013. Effect of fish sampling and tissue storage conditions in DNA quality: considerations for genomics studies. Revista de Investigación Marina, AZTI-Tecnalia, 20(6): 77-87.

3 Term of reference c) Review the adequacy of procedures for sample material collection, preservation and transfer to laboratory

Summary of provided information

The protocol provides a series of procedures for sample material collection, preservation and transfer to the laboratory. Concerning the type of sample to collect, the protocol advocates for fin or muscle tissue samples. The protocol provides a list of items that should be provided in a "sampling kit".

Observations

Although the protocol advocates use of muscle tissue or fin clip samples, there are alternative sample types that could be used if needed. For example, gill tissue usually results in higher DNA concentration but requires more time to collect. DNA concentration is not an issue here, since about 1ug of DNA can be obtained from about 20mg of tissue, and most genetic applications require much less. Therefore, which tissue to sample should be decided based on how easy it is to sample one or another. Although the protocol specifies that the sampling location should be provided, it is not clear if it refers to latitude/longitude or to larger areas (e.g. ICES area). We noted that the protocol does not provide specific instructions on how to collect the sample. Moreover, it is not clear what exactly is meant when it specifies that gloves should be changed "for each activity". Additionally, it is not clear what the action "Storing bulk and trace samples separately at all times." intended to avoid cross-contamination refers to.

Conclusions and recommendations

We recommend that:

- Clearer instructions about geographical location data needs should be provided;
- The sample collection protocol and, in particular, the actions to be taken between collection of each sample should be clearly described (e.g. after collecting one sample, a new kit should be started or all material should be rinsed with ethanol etc…);
- In particular, the actions to prevent cross-contaminations should be clearly specified and the controls that need to be introduced in order to detect if they occur.[2]
- The sample collection protocol should be revised to clarify/remove instructions and steps that are ambiguous (e.g. "*Storing bulk and trace samples separately at all times.*");
- If possible, a one-page sample collection protocol summary, laminated or printed e.g. on lexan plates should be produced. This would make it easier for controllers to follow

[2] This point was added as a response to the reviewers in Annex 3. Please see Annex 3 & 4 for more details.

instructions, and also for use under difficult working conditions. Ideally illustrations should be included to facilitate clarity.

4 Term of reference d) Review the adequacy of the genetic technique advocated in the IMP, provided that it should produce unequivocal evidence about species identification/misidentification

Summary of provided information

The IMP presents a number of known DNA based methods for species identification. Some of the methods described in the report are, as stated, "obsolete" for routine species identification and should be avoided unless for very specific applications, with validated routine genotyping systems already in place. This relates to AFLP, RFLP and RAPD, which generally are considered outdated solutions to DNA-based species identification. Thus, state of the art methods for unequivocal species identification are considered Sanger sequencing (DNA barcoding), qPCR (quantitative PCR) and related HRM (High Resolution Melt) analysis. qPCR and HRM are presented as the method of choice because they are fastest and more cost-effective.

Observations

The protocol is intended to be a "guide in the collection of samples from fisheries products for genetic analysis in the NAFO Regulatory Area by Inspectors from each NAFO contracting party (in the case of the EU, delegating to each member state)". Thus, there is no intention that the protocol provides detailed instructions for the application of the relevant genetic techniques. Instead, the protocol reviews the potential techniques which could be applied on the collected samples, which would be collected following the manual of procedures. Thus, the protocol advocates three main methods, qPCR, HRM and barcoding. New methods are under development (e.g. onsite high throughput sequencing), which require some tuning before routine application. In that sense it should be noted that the sampling protocol described in the manual would not need to be changed for application of these new methods, as once sufficient quantities of good quality DNA is obtained, virtually any DNA sequencing or amplification method can be implemented.

In terms of documentation, DNA barcoding represents the highest level of direct information on genetic differences between species and provides the necessary baseline for designing and validating assays and protocols for qPCR and HRM, as these latter indirect methods are one or two steps away from actual sequence information provided through DNA barcoding. Still qPCR and HRM can be tested and validated to forensic standards with unequivocal species identification. These methods have other attractive features, such as speed and volume of sample processing, which should be considered when implementing a method for routine analysis. Another benefit of qPCR and HRM compared to Sanger sequencing mentioned in the IMP: "both methods rely on a short DNA fragment (below 100 bp) and are thus less sensitive to DNA degradation." Thus, the method of choice, when dealing with samples or products where DNA degradation is likely to occur, should be considered, e.g. after long storage or processing (cooking).

Concerning barcoding, the protocol mentions that reference databases should be used for taxonomic assignment. The database mentioned is Genbank, which is not curated, meaning that a proportion of the available sequence information could relate to erroneous species identification. Alternatively, databases such as BOLD have been validated since they rely mostly on voucher specimens identified by taxonomic experts, thereby reducing the number of erroneous

sequences. Yet, BOLD is focused towards COI mtDNA sequences, thereby not allowing all types of DNA barcode information to be applied.

We note that for developing accurate qPCR and HRM assays and for obtaining accurate taxonomic identification from barcoding data, a prior verification of available reference databases is desirable due to errors in public reference databases and the possibility of intraspecific (DNA-sequence) variation.

Conclusions and recommendations

We suggest that the protocol clearly states that the decision on the methodology used for species identification should be taken depending on the question to be answered:

- Positive/negative species identification through screening of many samples could be performed using qPCR or HRM assays, which are faster and more cost effective than sequencing methods, or using Sanger sequencing;

- Unambiguous identification of the species to which a sample belongs should be done using Sanger sequencing, which allows taxonomic identification of samples without any prior information. This also generally holds for forensic validation of results obtained by qPCR or HRM.

5 Terms of reference e) Produce a report detailing the review of IMP and conclusions of WKGenMan on the basis of the ToRs a-d

We welcome the International Manual of Procedures (IMP) for fisheries product sampling for genetic analysis in support of the Northwest Atlantic Fisheries Organization (NAFO, - below referred to as 'the manual').

Detailed observations and recommendations for the different sections of the manual are listed under ToRs a) to d), specifically addressing what we perceive as text and instructions that could benefit from improvement.

In general, we observe that the manual could benefit in parts from editing and reviewing of the text to enhance clarity and ease of use for inspectors. Also, the introduction of illustrations would help inspectors to follow the instructions provided.

We deem it important to keep the often challenging working conditions of the inspectors on board of vessels in mind. It might be difficult for inspectors to follow the manual accurately in all steps.

Also, conditions on board may impede the ability of inspectors to follow instructions precisely. This is why accurate protocolling of all actions and deviations from instructions by inspectors is key, particularly in a control and enforcement context.

However, we trust that the envisioned pilot study (see also ToR a)) will provide for an opportunity to further assess the feasibility of the manual and enhance it, based on feedback by the participating inspectors.

In general, we recommend the inclusion of a 'Glossary' defining and clarifying terms used, which could be illustrated to facilitate usage.

In summary, we are convinced that the manual will strengthen the ability to monitor and control for compliance with species labelling. It is clearly in line with Article 13 of the Common Fisheries Policy Control Regulation (EC) No 1224/2009, and generally a valuable initiative to secure transfer of genetic and genomic approaches to routine applications in fisheries management. It should constitute a paradigm for similar manuals to follow, and will, if appropriately implemented and disseminated, contribute globally to a stronger fisheries control and enforcement capacity and a more efficient fight against Illegal, Unreported and Unregulated fishing.

Annex 1: List of participants

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Annex 2: Resolutions

Workshop on the manual for genetic sampling from fisheries products in the NAFO area (WKGenMan)

2020/WK/ASG04 The **Workshop on the manual for genetic sampling from fisheries products in the NAFO area (WKGenMan)**, in response to the EU-DGMARE request for ICES advice on the International **Man**ual of Procedures (IMP), an EU proposal to the Standing Committee of International Control (STACTIC) of the Northwest Atlantic Fisheries Organisation (NAFO) to guide the collection of samples from fisheries products for **gen**etic analysis, chaired by Jann Martinsohn (JRC, Italy) and Naiara Rodriguez-Ezpeleta (Spain), will work by correspondence until 12 January 2021 to address the request to review the IMP and to specifically:

Review the adequacy of the approach proposed in IMP for collection of genetic material for sampling of fish on board of vessels for species identification;

b) Evaluate feasibility and recommend a method for sample preservation that will be effective at maintaining sample integrity in the NAFO Regulatory Area fisheries, where at least 7 days are needed from sample collection to delivery at a laboratory facility;

c) Review the adequacy of procedures for sample material collection, preservation and transfer to laboratory;

d) Review the adequacy of the genetic technique advocated in the IMP, provided that it should produce unequivocal evidence about species identification/misidentification; and,

e) Produce a report detailing the review of IMP and conclusions of WKGenMan on the basis of the ToRs a-d; and,

To carry out this work, WKGenMan, a Core Group of members from the ICES Working Group on Application of Genetics in Fisheries and Aquaculture (WGAGFA), including an invited expert from NAFO, will work by correspondence. WKGenMan will report by 12 January for the attention of the ASG, ACOM and SCICOM.

Supporting information

Priority	High, in response to a specific request from the EU Commision to ICES to prepare a review and advice on the International Manual of Procedures (IMP). The advice should provide the scientific knowledge basis to assess the IMP based on the ToRs above.
Scientific justification	The IMP is an EU proposal to the Standing Committee of International Control (STACTIC) of the Northwest Atlantic Fisheries Organisation (NAFO) with the objective of guiding fishing inspectors in carrying out their assignments, particularly the assignments directly derived from implementation of Article 35 of the NAFO Control and Enforcement Measures (CEM). The NAFO CEM includes provisions for DNA analysis in an effort to develop a solid approach to combat issues related with species misidentification. The IMP is a tool to operationalise the use of genetics in combating fish fraud and IUU fishing in the NAFO Regulatory Area (NRA), notably by providing the guidance and setting the rules for the collection of samples of fishing products by any fishing Inspectors of NAFO contracting Parties operating in the NRA. The IMP should encompass (1) genetic tools and techniques and (2) techniques and approaches for the collection of genetic material that, at present, provide the most accurate results and represent the best practices on the matter. The IMP should be a tool that is validated and endorsed by the ICES scientific community.

Resource requirements	ICES Secretariat support and Advisory process
Participants	The Core Group is expected to comprise 5-6 members and an expert familiar with sampling in the NAFO regulatory area. Other members of WGAGFA will be consulted.
Secretariat facilities	Secretariat support, web conferences.
Financial	Covered by DG MARE special requests to ICES
Linkages to advisory committees	ACOM
Linkages to other committees or groups	WGAGFA, SCICOM, ASG
Linkages to other organizations	EU DG MARE, NAFO

Annex 3: Review Group for WKGenMan Report 2021

Consensus report from Review Group for the ICES WKGenMan- - WGAGFA Report on the ICES IMP "FISH PRODUCTS SAMPLING FOR DNA TESTING"

The two members of the Review Group John Hyde (USA) and Fausto Tinti (Italy) prepared separate reviews of the WKGenMan- - WGAGFA Report, and discussed the report and their reviews on January 14, 15 and 18 during three 1 hour web-meetings. Key points emerging from the discussion included:

Overall review

We recognize the important contribution of the working group report on the development of a standardized manual for sampling procedures for genetic monitoring of fishery products for species identification (IMP). Given the broad scope of implementation of the sampling program and potential uses of the obtained samples, it is of utmost importance to ensure the IMP is properly reviewed. Potential uses of the sampling range from logbook and observer validation studies aimed to improve fishery statistics to forensic tracking of intentionally mislabelled seafood that may result in legal cases. In addition to the requirement to monitor species labelling, the availability of properly preserved and documented samples in an accessible archive has the potential for countless ancillary studies that have the potential to improve fishery management (e.g. identification of cryptic species, studies of stock structure and traceability of geographical origin). Below we comment on the specifics to the working group report, highlighting shared comments between reviewers and perceived strengths and weaknesses of the report.

Shared Comments

Overall both reviewers identified many of the same strengths and weaknesses of the report (see below), specific details can be found below and within individual reports. In general, we feel that the report did a good job identifying strengths and weaknesses of the IMP. The overarching theme between the reviewers and the report itself is that the IMP could benefit from increased detail and clarity to ensure uniform and successful sample and data collection. Such detail and clarity are especially important given the possible use of samples for forensic identification that may result in legal actions. Though non-forensic use of samples may be okay with a lower level of documentation, every effort should be made to ensure sampling protocols and documentation are of the same highest standard for all sampling. It should not be understated that proper sampling methodology and documentation are the foundation for a successful monitoring program.

Strengths:

- One of the stronger points highlighted in the report is the need for an initial pilot project to test protocols and overall implementation. This is essential as undoubtedly problems and novel workflows will be identified that can then be used to update the IMP.
- The report properly identified a number of areas where increased details on sampling procedures need to be provided and suggested simple weather proof instructions be created to aid samplers for reference.

- Overall review of genetic methodology and reference data was strong and highlighted a number of important points including advantages and disadvantages of individual methods.

Weaknesses:

- Though the report identified a number of gaps in regard to data collection and sampling protocols there are still several critical areas that were insufficiently covered. These areas include: discussion of data collection requirements and handling procedures for forensic uses; detailing specific sampling protocols to ensure proper sample preservation; handling and tracking of samples post-collection.
- The report insufficiently covered sample preservation protocols and the guidance that preservative "covers the sample" and that "samples should not be too large" needs to be revisited. For proper preservation the preservative: sample volume ratio must be at least 5:1, specific guidance to this point is absolutely necessary.
- More detail needs to be provided for sampling so as to avoid cross contamination issues.
- The report fails to adequately discuss the limitations of mitochondrial DNA (i.e. COI Barcode) data to identify species. These limitations include both the somewhat rare occurrence of hybrids where the maternally inherited marker would be unable to detect this as well as for taxonomic groups where the marker provides insufficient resolution.

Review by John Hyde, Supervisory Geneticist, NOAA – National Marine Fisheries Service, USA.

The ICES WKGenMan working group was tasked with review of the IMP manual for genetic sampling and species identification from NAFO Regulatory Area to combat mislabelling of seafood. Specific to their task was to address the following Terms of Reference (ToR):

I present below my review of the provided information by each identified ToR:

a) Review the adequacy of the approach proposed in IMP for collection of genetic material for sampling of fish on board of vessels for species identification.

The report describes the overall goals and approach of the IMP and highlights among them several key aspects. The overarching protocols and record keeping depend upon the intended use of the samples with the use for law enforcement forensics requiring a significantly higher investment of time and resources than for routine monitoring of catch composition. For law enforcement forensics there is required attention to several key areas: sampling method and design to ensure adequate and representative sampling as well as ensuring sampling methods eliminate the chance of cross contamination between samples; chain of custody documents and procedures to track samples and ensure their integrity both in the field and lab; documentation of training and protocols used for both field and lab work; assurance that identifications are unambiguous, typically this requires that curated reference samples or DNA sequences from all possible species are queried and that the methods used are capable of differentiating all possible taxa. As noted, there may be some limitations on the extent of sampling possible dependent upon the location of sampling (i.e. at sea, dockside, market) as well as the disposition of the catch (i.e. fresh whole fish, frozen boxes of fillets).

A key aspect of the report is highlighting the need for a pilot-level project to field test the protocols, identify potential issues, and refine the manual and associated protocols based on the pilot project findings. This is an important point as it is inevitable that limitations will exist and

require protocol modification. For this reason, the IMP should still be treated as a draft until the pilot project is completed and feedback analyzed.

b) Evaluate feasibility and recommend a method for sample preservation that will be effective at maintaining sample integrity in the NAFO Regulatory Area fisheries, where at least 7 days are needed from sample collection to delivery at a laboratory facility.

The key to success of programs such as that proposed is that sample quality/integrity is maintained until analyzed in a lab and ideally longer in case reanalysis is warranted for QA/QC procedures or verification using different genetic methodologies. At the core of this requirement is collection of samples in as fresh a state as possible (ideally at time of capture) and then immediate preservation to significantly retard or eliminate further DNA degradation. As pointed out in the report, the goal should not be stabilization for 5-7 days but rather focus on methods that may preserve the samples indefinitely. Luckily in this case, there is significant literature and experience dealing with field preservation of tissue samples for genetic purposes.

There is discussion both on the size of samples to collect and the use of preservatives, these issues need to be focused on a bit more. Ensuring that the preservative "covers the sample" and that "samples should not be too large" is insufficient guidance that is bound to lead to failure. Typical guidance for both proposed preservatives (96% ethanol and RNA*later*™) are for a 5:1 or greater ratio of preservative: sample and as mentioned in the report, for longer-term preservation the preservative should be replaced after ~24 hours as initial preservative concentration will be diluted by the water present in the tissue. A range of tissue sizes of 2-4.5 cm^3 are given which are then to be subdivided into 3 pieces and stored in separate vials. Taking the higher end amount would require a total volume of ~9ml (1.5 cm^3 tissue plus 7.5ml preservative) in a vial for each subsample. Ideally a standardized method is adopted with a single directed maximum size of sample and standard vials filled with sufficient preservative such that adding too much tissue would overflow the vial (acting as an indicator to prevent insufficient preservative). It should also be noted that forethought needs to be given to long-term accession and storage of samples. Given sample collections typically grow in perpetuity, vial size, labelling, specialized storage conditions (i.e. freezers, flammable lockers) all need to be considered to avoid significant storage limitation in the near future.

c) Review the adequacy of procedures for sample material collection, preservation and transfer to laboratory.

The report identifies a number of shortcomings from the IMP, specifically with instructions both for sample collection and data entry. These basic details are key to successfully implementing a genetic sampling program. As discussed above, the end use of the samples dictates the degree of detail and documented protocols required. Regardless, sufficient detail needs to be supplied such that protocols are standardized across users. Careful attention is required to ensure that all required data are collected and entered in a legible and permanent manner on vials and data sheets.

Some discussion should be given to sampling such that samples are not cross contaminated. Blood and slime can easily be cross contaminated between fish so collection of muscle may be a preferred tissue type over fin unless the sample is cleaned off well. Similarly, as mentioned other tissues can be taken besides the recommended muscle or fin.

The report suggested creating simple laminated instructions that can be referenced while at sea. This is a great suggestion, especially during early implementation of the program and training of new samplers.

d) Review the adequacy of the genetic technique advocated in the IMP, provided that it should produce unequivocal evidence about species identification/misidentification.

The report discusses some shortcomings in the IMP in regard to outdated identification protocols and highlights newer and upcoming methods. As stated, the IMP does not provide specific guidance on the technique to use for identification purposes which is likely driven by sample processing time, number of samples, and end use that may require more complex protocols (i.e. forensics). Overall the report did a good job of summarizing methods and needed changes.

Discussion is provided on how Barcoding is the standard method to verify species identification, which is generally true but it has some caveats. As discussed in the report, a curated and accurate sample database is required to assign identifications. To this end the use of GenBANK was discouraged due to known sample identification issues and rather the Barcode of Life Database (BOLD) was encouraged.

Additional discussion should be provided to ensure that other caveats of the COI Barcode method are understood. These include: inadequate taxonomic resolution at the COI gene; the haploid nature of mitochondrial DNA prevents identification of hybrids; reference sequence must be available for all the species that may be encountered in order to assign identification.

e) Produce a report detailing the review of IMP and conclusions of WKGenMan on the basis of the ToRs a-d.

The overall report of the review and conclusions of WKGenMan were well described with important suggestions on implementation. In general, there is encouragement to improve clarity and instructions with illustrations/figures as well as a glossary. All the points made are important and should be addressed to improve the IMP.

Review by Fausto Tinti, Associate Professor in Zoology, Dept. Biological, Geological and Environmental Sciences, University of Bologna, Italy.

I reviewed the WKGenMan report according to the ToRs identified by ICES.

ToR a) Review the adequacy of the approach proposed in IMP for collection of genetic material for sampling of fish on board of vessels for species identification.

WKGenMan report adequately defines and corroborates the framework in which the approach of IMP should be implemented and will be operative that is “the first time that DNA-techniques will be used at a large scale in European waters (and beyond) to monitor for compliance with species labelling rules”, in line with the Article 13 of the Common Fisheries Policy Control Regulation and supporting legal investigations on mislabelling and commercial frauds. The intended use of IMP should go far beyond “the mere information”, and it is expected to reach an efficiency and reliability for providing evidence in a legal control and enforcement framework. Statistical and methodological requisites of the approach have been analyzed correctly by WKGenMan and gaps and weaknesses identified and addressed by recommendations and suggestions.

Overall, the WKGenMan report appropriately reviewed the scientific (including a better statistical approach) and technical matters and specific recommendations on the need for dedicated training of inspectors and for verifying/optimizing the protocols through field pilot tests are

critical and effectively improve the IMP. In my opinion the WKGenMan report **weakly reviewed the forensic requisites, duties and responsibilities that if not well identified and addressed also in the sampling and handling of samples they could allow the risk to prevent the full forensic exploitation of the downstream DNA-based evidence in the event of legal investigations and court cases.**

ToR b) Evaluate feasibility and recommend a method for sample preservation that will be effective at maintaining sample integrity in the NAFO Regulatory Area fisheries, where at least 7 days are needed from sample collection to delivery at a laboratory facility.

The WKGenMan analysis is only partially adequate and correct because addressed correctly several technical requisites of the IMP related to sampling and delivering of samples to the lab analysis, on which I fully agree with WKGenMan. Important recommendations were defined by the WGAGFA to reduce/eliminate the risk of DNA degradation such as the immediate tissue sampling when fish are landed on the deck, avoiding retard and handling operations at RT. Such technical improvement, if combined with a better standardized sample preservation, will allow long-term (more and more than 7 days) tissue quality and potentially an indefinite availability of the DNAs suitable for a wide array of genetic applications and future scientific and forensic demands beyond the species identification. On this matter, I consider **the revision of the "sample preservation" procedures weak and only partially correct**. The critical condition either for a short-term or long-term preservation - and thus suitable for end-uses – of samples is the volume ratio tissue: storage buffer [1, 2]. This ratio should allow a full penetration of storage buffer into the wall tissue sample and is independent from the sample dimension. For both storage methods (i.e. ethanol96% and RNA*later*™) it is misleading and unclear the report recommendation "it is crucial that the liquid covers the sample…". If an adequate volume ratio (e.g. from 1:5 to 1:20) was not applied, the risk that tissue sample cannot be fully penetrated by preservation buffer and consequently tissue rot and enzymatic DNA degradation are highly risky. Therefore, both sample dimension and kit/disposable should be better and deeply defined according to the volume ratio tissue: storage buffer adequate for assuring correct preservation of tissue and nucleic acids. Smaller dimensions of the tissue samples (20 mg of well-preserved tissue is enough for extracting huge amounts of genomic DNAs and largely enough for PCR-based downstream applications) will likely allow an easier handling of large number of tissue samples that need to be catalogued and stored onboard in the refrigerated conditions (i.e. the use of microtubes with 1-2 mL of preservative for each sample) and minor costs. Related to the delivering of samples and with respect to the forensic framework, **the transferring of sample could be critical because samples and sample boxes should be handled under correct conditions (temperature and labelling) and avoiding the risk of manipulation (intentional or accidental).** The proposal of an adequate and safe protocol for transferring of samples to laboratory should be recommended.

[1] Nagy 2010. A hands-on overview of tissue preservation methods for molecular genetic analyses. Org Divers Evol 10: 91–105, https://doi.org/10.1007/s13127-010-0012-4

[2] Stein et al. 2013. Evaluating Ethanol-based Sample Preservation to Facilitate Use of DNA Barcoding in Routine Freshwater Biomonitoring Programs Using Benthic Macroinvertebrates. PLoS ONE 8: e51273. https://doi.org/10.1371/journal.pone.0051273.

ToR c) Review the adequacy of procedures for sample material collection, preservation and transfer to laboratory.

The WKGenMan report identified some critical gaps and poorness of the sampling protocol in term of collected associate data to the sample (e.g. the lack of a clear identification of collecting site and associated information as ICES or FAO area) and of detailed instructions for observers for sampling procedures to avoid cross-contamination. WGAGFA properly recommend how fill

them collecting data as geographical coordinate and detailing better the handling procedures during the sampling. However, **additional data as sampling date, depth, as well as the stage, total size, or the type of fish product (e.g. fresh and frozen fish or fillet or portion of the fish) need to be collected and most importantly univocally associated to the tissue sample and to DNA-based evidence of correct or mislabelling.** More info could be retrieved and rescued in the event of legal investigations or court cases (which likely will occur several months later), more efficient and reliable will be the IMP for this purpose.

Because the huge number of samples that will be collected in a very narrow time window the IMP should also address advices and recommendations on how to organize and manage the archive either for the logistic or data mining.

ToR d) Review the adequacy of the genetic technique advocated in the IMP, provided that it should produce unequivocal evidence about species identification/ misidentification

The WKGenMan report identified the most recent and efficient molecular methods and DNA-based genetic technologies to address species identification along with the fishery supply chain (targeting non-degraded or weakly-degraded DNA fragments). The need of referenced databases (e.g. the universal Barcoding of life database and the FishBold initiative therein in the case of use of Sanger DNA sequencing) as integral part of the technologies is highlighted by the technical analysis in the report.

Two main gaps were not addressed by the WKGenMan report.

First, **the uncommon but existing cases of fish hybrid species** (e.g. the hybrid flatfishes Isopsetta isolepis X Parophrys vetulus, [3] and Inopsetta ischyra [4]) challenges and affected some genetic application [5]. This should be included as a potential caveat of the mtDNA-based techniques and the integration with nuclear DNA and/or morphological analyses should be recommended. Second, non-adequate storage and processing conditions, in particular for long-term storage of samples, can lead also to **DNA nucleotide sequence alteration reducing sensitivity of PCR-based and sequencing methods** [6] and this should be accounted in the IMP at least to recommend the need of a well preservation of tissue samples.

[3] Garrett & Buth 2005. A New Intergeneric Hybrid Flatfish (Pleuronectiformes: Pleuronectidae) from Puget Sound and Adjacent Waters, Copeia 2005: 673-677, https://doi.org/10.1643/CI-04-344R.

[4] Garrett et al. 2007. The Hybrid Sole Inopsetta ischyra (Teleostei: Pleuronectiformes: Pleuronectidae): Hybrid or Biological Species?. Transactions of the American Fisheries Society, 136: 460-468, https://doi.org/10.1577/T06-092.1.

[5] Amorim et al. 2020. Species assignment in forensics and the challenge of hybrids. Forensic Science International: Genetics, 48: 102333, https://doi.org/10.1016/j.fsigen.2020.102333.

[6] Pecoraro et al. 2020. Canning Processes Reduce the DNA-Based Traceability of Commercial Tropical Tunas. Foods 9:1372, https://doi.org/10.3390/foods9101372.

ToR d e) Produce a report detailing the review of IMP and conclusions of WKGenMan on the basis of the ToRs a-d

Overall, the WKGenMan report identifies the need to ameliorate several aspects of IMP for sampling fish and fish products for DNA analyses, suggesting a list of several effective and technical/operative improvements. Horizontal tasks as the training and skilling of inspectors, pilot testing of the procedures, the adoption of a common-in-the-field technical glossary and the attention to make sampling instructions clearer and easier to be followed are the most relevant recommendations of the report.

Annex 4: Expert Response to Reviewers

We would like to thank John Hyde and Fausto Tinti for reviewing of our advice on the International Manual of Procedures for genetic sampling (IMP) and for providing a consensus report highlighting shared comments, strengths, and weaknesses on our report.

Here, we focus on the weaknesses raised and explain where and how the WKGENMAN report was modified to accommodate them or provide reasons when no modifications were considered necessary.

- ***Though the report identified a number of gaps in regard to data collection and sampling protocols there are still several critical areas that were insufficiently covered. These areas include: discussion of data collection requirements and handling procedures for forensic uses; detailing specific sampling protocols to ensure proper sample preservation; handling and tracking of samples post-collection.***

However, ***additional data as sampling date, depth, as well as the stage, total size, or the type of fish product (e.g. fresh and frozen fish or fillet or portion of the fish) need to be collected and most importantly univocally associated to the tissue sample and to DNA-based evidence of correct or mislabeling****. More info could be retrieved and rescued in the event of legal investigations or court cases (which likely will occur several months later), more efficient and reliable will be the IMP for this purpose.*

Because the huge number of samples that will be collected in a very narrow time window the IMP should also address advices and recommendations on how to organize and manage the archive either for the logistic or data mining.

We, WKGenMan, observe: We believe that much of the information mentioned here will be documented in logbooks. Particularly we find it important to keep in mind that the IMP must be a guideline that enables efficient monitoring, control and enforcement, while remaining feasible for inspectors, who work often under very difficult conditions. Rather than coming forward too many suggestions for data to be collected, the recommended pilot study will provide for the opportunity to test the IMP under real conditions.

- **The report insufficiently covered sample preservation protocols and the guidance that preservative "covers the sample" and that "samples should not be too large" needs to be revisited. For proper preservation the preservative:sample volume ratio must be at least 5:1, specific guidance to this point is absolutely necessary.**

We, WKGenMan, observe: We think the reviewers are correct. After revisiting the protocol and our report, we have noticed that the tissue volume suggested is too large to ensure penetration of the preservative into the sample. We thus have modified the WKGenMan report to suggest

that the sample collected should not be larger than 1.5cm^3 so that each subsample is of about 0.5 cm^3.

- ***More detail needs to be provided for sampling so as to avoid cross contamination issues.***

We, WKGenMan, observe: We agree with the reviewers that the issue of avoiding cross-contamination was overlooked in the protocol. We tried to specify this in ToR c, where we point to a lack of clarity. In order to make this clearer, we have added a new point to the recommendations derived from ToR c.

- ***The report fails to adequately discuss the limitations of mitochondrial DNA (i.e. COI Barcode) data to identify species. These limitations include both the somewhat rare occurrence of hybrids where the maternally inherited marker would be unable to detect this as well as for taxonomic groups where the marker provides insufficient resolution.***

We, WKGenMan, observe: *We are aware that some phenomena such as hybridization and introgression between species can result in genetic assays not providing accurate species identification. However, as we state in our ToR d, the aim of the protocol is not to provide detailed instructions for the application of the relevant genetic techniques, but to provide a solid and feasible protocol that ensures the samples collected are suitable for genetic identification. For this reason, we believe that there is no need to enter into these details as the specific assay to be developed for each case should consider specific knowledge of each species.*