The use of environmental DNA methods for detection of WOAH listed aquatic animal diseases

A discussion paper developed by the WOAH Aquatic Animal Health Standards Commission (Aquatic Animals Commission).

1.   Summary

The monitoring of aquatic systems using environmental DNA (eDNA) is a rapidly advancing research field that will provide opportunities for cost-effective, non-destructive methods to screen for pathogenic agents, including those of wild aquatic populations where samples may be difficult or undesirable to obtain.

The Aquatic Animals Commission is aware that eDNA methods are being applied for detecting the causative agents of several WOAH listed diseases. As these methods are available and currently in use, the Commission has agreed that it would be advisable for guidance to be provided on appropriate application of eDNA methods and potential limitations.

The Commission notes that, as accurate estimates of diagnostic performance are not available for designing surveillance programmes using eDNA assays, data obtained from eDNA methods are not suitable to support declarations of freedom from listed diseases. Confirmation of infection with listed diseases could also not be made using eDNA methods because a positive result does not demonstrate that a susceptible host animal(s) is infected.

Positive eDNA results could, however, provide evidence amounting to suspicion of infection such as the presence of the pathogen DNA/RNA in the sample. This application of eDNA methods may be particularly useful for the monitoring of high-value or rare animals as an alternative to collection of tissue samples. It has a potential role in early detection of disease incursion in wild populations or under circumstances when infection is not likely to result in observable clinical signs. However, following suspicion, based on positive eDNA, samples obtained directly from aquatic animals need to be tested as described in the relevant disease-specific chapters of the *Manual of Diagnostic Tests for Aquatic Animals* (*Aquatic Manual*) to confirm or exclude the case.

The application of eDNA methods for a given purpose should be considered carefully. Methods should be chosen with consideration given to all relevant factors including the surveillance objective, the target pathogen, the reliability of the method, and the environment to be sampled. It is important that the implications of positive results be considered in advance of applying an eDNA method as any positive results may require that surveys involving direct sampling and testing of susceptible animal be conducted to confirm or exclude a suspect case. eDNA methods will not be an appropriate choice for many aquatic animal disease surveillance purposes.

This document is intended to explore the potential use of eDNA methods with respect to the standards of the WOAH *Aquatic Animal Health Code* (*Aquatic Code*) and *Aquatic Manual* and to outline benefits and limitations.

The use of an eDNA method for the detection of *Gyrodactylus salaris* has been included in *Aquatic Manual* Chapter 2.3.3 Infection with *Gyrodactylus salaris*[1]. The inclusion of this method conforms with the conclusions of this discussion paper.

2.   Definitions for eDNA

Numerous definitions for eDNA exist (e.g. Bass *et al*., 2015; Diaz-Ferguson & Moyer, 2014; Thomsen & Willerslev, 2015). Most definitions regard eDNA as detectable short DNA/RNA fragments from a living organism derived from cellular components or fluids secreted into the abiotic components of surrounding environment (i.e. water, air, sediments).

For the purposes of this document, we define eDNA as: "nucleic acids of pathogenic agents extracted from 'true' environmental samples (such as water, soil, sediment, biofilm)". Direct host-derived material such as faeces, sloughed cells and mucous, are excluded from this definition. Once extracted from the environmental sample, target eDNA fragments can be detected using a variety of molecular methods (Diaz-Ferguson & Moyer, 2014). Furthermore, eDNA can be sequenced directly as metagenetic libraries or after PCR amplification of specific target gene regions (Bass *et al*., 2015).

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1   https://www.woah.org/fileadmin/Home/eng/Health_standards/aahm/current/2.3.03_G_salaris.pdf

The actual performance of eDNA based detection depends on the sample collection and processing methodology (e.g. volume filtered, presence and removal of PCR inhibitors), biological processes (e.g. rates of shedding, temporal variation) and abiotic factors (analyte degradation, hydrodynamic factors). It is important to evaluate these factors empirically so that the results can be properly interpreted. It is only with a clear understanding of how these factors influence the probability of pathogenic agent detection that eDNA-based detection can be used reliably in a variety of settings (Brunner, 2020).

3. Objectives

This paper considers i) the benefits and ii) limitations of eDNA pathogenic agent detection methods, iii) validation of eDNA methods, iv) the conditions for inclusion of an eDNA method in a disease-specific chapter of the *Aquatic Manual* and v) use of eDNA evidence as diagnostic criteria.

4. Review of published eDNA methods for the detection of aquatic animal pathogenic agents

A literature review was undertaken to assess the application of eDNA methods for the detection and study of pathogens and parasites of aquatic animals. Thirty-three publications reporting the use of eDNA to detect thirteen WOAH listed pathogenic agents were identified (see Appendix 1, Table 1 for details). Methods have been developed for the detection of the causative agents of WOAH listed pathogenic agents of amphibians, crustaceans, fish and molluscs. The majority of publications concern the detection of the listed pathogenic agents in wild aquatic animal populations, notably infection with *Aphanomyces astaci*, infection with *Batrachochytrium dendrobatidis*, infection with *B. salamandrivorans*, infection with *Ranavirus* species, infection with *G. salaris*.

A further thirteen publications were found that targeted other specific pathogenic agents (e.g. *Mikrocytos mackini*), groups of pathogenic agents (e.g. of ornamental fish) or applied eDNA methods to broader areas of study (e.g. water-borne transmission of viruses) (see Appendix 1, Table 2 for details).

5. Benefits of eDNA methods for the detection of aquatic animal pathogenic agents

eDNA detection is a promising tool that can be used to complement direct sampling of aquatic animals for surveillance. eDNA methods offer some benefits compared to direct sampling and testing of aquatic animals, including, but not limited, to the following:

1. eDNA methods do not require intrusive or lethal sampling of aquatic animal hosts. They may be particularly useful for rare or valuable aquatic animals, or difficult to collect wild animals (e.g. Rusch *et al*., 2018).

2. eDNA methods do not require handling of animals, avoiding the stress associated with obtaining non-destructive tissue samples (Brunner, 2020).

3. Sample collection and sample processing time and associated costs may be reduced substantially compared to collection and processing of individual animal samples (Rusch *et al*., 2018).

4. As environmental samples may contain analyte from the entire, or a large percentage of a target captive population, many fewer samples may be required to detect a pathogenic agent (compared to individual animal samples), even when diagnostic sensitivity of the eDNA method is low (Brunner, 2020).

5. The same environmental sample can be analysed for the presence of host species (e.g. see Rusch *et al.*, 2018) and multiple pathogens.

6. eDNA methods could be used for assessment of potential introduction pathways where sampling of hosts is not possible (e.g. ballast water).

6. Limitations of eDNA methods

Limitations to the application of eDNA based pathogenic agent detection include, but are not limited to, the following:

1. Very little target pathogen DNA/RNA may be available in the environmental sample due to dilution in the environment and degradation of nucleic acids. This may negatively impact the sensitivity of the method (Brunner, 2020).

2. The concentration of target DNA/RNA in an environmental sample will vary due a range of factors such as host density, prevalence and intensity of infection, sampling method (e.g. for water volume sampled, filter pore size, storage conditions) and environmental conditions (e.g. amount of organic matter). Sensitivity of eDNA methods may, therefore, vary more between localities, surveys undertaken at different time points and target taxa than direct sampling and testing of animal tissues (Brunner, 2020).

3. There are formal frameworks to assess diagnostic performance of tests using animal-derived samples, but these have not been developed for eDNA methods. This means that the design of surveys to demonstrate freedom from infection using eDNA methods is problematic.

4. A positive detection of target pathogen DNA/RNA in an environmental sample may be more likely to result from a source of contamination not representative of viable pathogen (e.g. inactivated pathogen from heat treated products) compared with animal-derived samples. Similarly, it may not indicate infection of a host animal with the target pathogenic agent.

7. Validation of eDNA methods

There is an increasing likelihood that disease management decisions will be made based on results from eDNA studies. It is thus imperative that data generated by eDNA studies is reliable, defendable and executed with high quality assurance standards (Klymus *et al.*, 2019). Empirical validation of eDNA-based pathogen detection should focus on understanding the causes and consequences of variation in test characteristics across sampling conditions and needs to take into consideration a clear understanding of what is being sampled/assayed for in the case of each pathogen of interest.

Chapter 1.1.2. of the *Aquatic Manual* describes the principles and methods of validation of diagnostic assays for infectious diseases. The recommendations of this chapter are intended for diagnostic testing of animal-derived samples; however, the principles and many of the methods are applicable to eDNA methods. It is recommended that the general principles and methods of Chapter 1.1.2. be applied to the validation of eDNA detection methods for WOAH listed diseases. It should be noted that the process of sample collection, the concentration of target DNA, the DNA extraction, the sensitivity and other performance (indicators) should be emphasised and validated.

Design and reporting standards are available for diagnostic accuracy studies for methods utilising aquatic animal-derived samples (e.g. Laurin *et al.*, 2018). Many of the design and reporting considerations are also applicable to eDNA methods and it is recommended that these standards be applied for eDNA diagnostic accuracy studies.

Additional to the guidance described above, design and reporting considerations have been published specifically for eDNA methods (e.g. Doyle & Uthicke, 2020; Goldberg *et al.*, 2016; Klymus *et al.*, 2019). Many of these studies report on considerations for detection of macro-organisms rather than pathogenic agents; however, the considerations are generally relevant for eDNA detection methods for pathogenic agents. This guidance will be of particular use for the field collection, processing and preservation of eDNA samples.

8. **Minimum requirements for inclusion of an eDNA method in the *Aquatic Manual***

It is recognised that the validation pathway described in Chapter 1.1.2. of the *Aquatic Manual* and the design and reporting standards described by Laurin *et al*., 2018 (see above) are not met by many diagnostic methods currently included in the *Aquatic Manual*. Indeed, many assays included in the *Aquatic Manual* may be validated only to level 1 or 2 of the validation pathway described in Chapter 1.1.2. of the *Aquatic Manual*.

For this reason, the Commission proposes that the following minimum reporting requirements be met for an eDNA method to be considered for inclusion in the *Aquatic Manual* [Adapted from Goldberg *et al*., (2016)]:

1. The intended purpose or application of the assay or protocol needs to be clearly defined (note that appropriate purposes of use for eDNA methods in the context of WOAH standards are discussed further in section 9).

2. Description of sample collection methods and precautions taken to eliminate contamination, including collection volume, container material, negative controls, number of replicates and sampling locations/depth.

3. Description of the methods used to concentrate the target DNA/RNA (precipitation/filtration), filter type (if applicable) and filtering location (e.g. in the field).

4. Description of sample preservation and storage (method, temperature, duration).

5. Description of the DNA/RNA extraction process including protocol adjustments, contamination precautions, negative controls, and internal positive controls.

6. Description of the molecular detection method and optimisation according to (Bustin *et al*., 2009). Furthermore, assays should be validated (Level 1) in an environmental matrix according to its purpose of use.

9. **Potential application of eDNA detection methods in the disease-specific chapters of the *Aquatic Manual***

The disease-specific chapters of the *Aquatic Manual* recommend tests to identify suspect cases and to confirm suspicion for apparently healthy (or those of unknown health status) and clinically affected animals. Apparently healthy populations may fall under suspicion, and therefore be sampled, if there is an epidemiological link(s) to an infected population. Geographic proximity to, or movement of aquatic animals or aquatic animal products or equipment, etc., from a known infected population equate to an epidemiological link. Alternatively, healthy populations are sampled in surveys to demonstrate freedom.

The following points describe the suitability of evidence from eDNA detection methods for inclusion as case definition criteria in section 6 of the disease-specific chapters of the *Aquatic Manual.*

a) Apparently healthy animals

   i) Definition of suspect case in a population of apparently healthy animals

   Suitable as a criterion. A positive result obtained from an eDNA method recommended in the *Aquatic Manual* is considered to provide adequate evidence to be included as a criterion for a suspect case when known susceptible species exist in the environment from which the sample was taken.

   ii) Definition of confirmed case in apparently healthy animals

   Not suitable as a criterion. A positive result obtained from an eDNA method recommended in the *Aquatic Manual* is not considered to provide appropriate evidence to confirm a case in apparently healthy animals. Methods utilising animal-derived samples are considered more appropriate for criteria to confirm a case. Evidence to confirm a case in apparently healthy animals must meet the requirements of Section 6.1.2. of the relevant disease-specific chapter of the *Aquatic Manual*. eDNA evidence will not be included as a criterion within this section.

b) Clinically affected animals

    i) Definition of a suspect case in clinically affected animals

Suitable as a criterion. Taking an environmental sample to investigate the cause of disease in a population of clinically affected animals is not generally recommended as samples from clinically affected animals are more likely to lead to pathogenic agent detection and are more suitable for disease investigation. However, under some circumstances, an eDNA method may detect a pathogenic agent and lead to the recognition of previously unobserved or unassociated clinical signs of disease. In these circumstances, a positive result obtained from an eDNA method recommended in the *Aquatic Manual* is considered to provide adequate evidence to be included as a criterion for a suspect case.

    ii) Definition of confirmed case

Not suitable as a criterion. A positive result from an eDNA method recommended in the *Aquatic Manual* would not be included as a criterion for the confirmation of a pathogenic agent in clinically affected animals (or apparently healthy animals, see point 9.a.ii above). Any positive eDNA test would require further investigation involving the collection and testing of animal tissues as stipulated in the relevant disease-specific chapter of the *Aquatic Manual*. Evidence to confirm a case in clinically affected animals must meet the requirements of Section 6.2.2. of the relevant disease-specific chapter of the *Aquatic Manual*. eDNA evidence will not be included as a criterion within this section.

10. Discussion

The key limitations of eDNA is the lack of validation and diagnostic performance data, meaning that negative results cannot be used to demonstrate disease freedom and positive results always require confirmation using animal samples (Brunner, 2020). Nevertheless, there are circumstances where the advantages of environmental, over animal, sampling means that eDNA approaches can be usefully integrated into a surveillance programme.

A country or zone claiming freedom from a specified pathogenic agent(s) are required to have in place an early detection system for disease incursion. Farmer reporting of morbidity and mortality is a key component of an early detection system. Farmed populations can act as sentinels for wild populations only if they are epidemiologically connected (i.e. through shared water). Otherwise, active surveillance in wild populations is required as morbidity or mortality is unlikely to be reported (especially as dead or dying animals are likely to be quickly scavenged or predated). Animal sampling of wild populations can present considerable logistical challenges, especially if populations are remote, sparse or if low numbers make destructive sampling undesirable. eDNA based pathogenic agent detection methods overcome many of the challenges of sampling wild aquatic animals (Kamoroff & Goldberg, 2017; Trebitz *et al.*, 2017).

Infection with some listed pathogenic agents, under certain conditions or in some host species, will not invariably cause detectable clinical signs. Early detection systems that rely on observations by farmers (or others) of mortality or morbidity are ineffective in these circumstances and active surveillance would be required. Sampling farmed animals on a frequent basis, and at a level to detect a low prevalence, presents considerable logistical challenges and the cost is likely to be unacceptable. eDNA methods can offer a viable alternative (Trujillo-Gonzalez *et al.*, 2019a) for active surveillance for pathogens which may not reliably cause observable clinical signs. They have the additional advantage that the sample will contain analyte from a large percentage, if not the entire, captive population. Thus relatively few environmental, compared with animal samples, are needed (provided sufficient DNA/RNA can be extracted).

11. Conclusions

1. eDNA methods may have utility for enhancing passive surveillance systems for early detection; particularly in circumstances where conditions are not conducive to clinical expression of disease, or populations are not under sufficient observation to detect clinical disease should it occur.

2. eDNA methods may have utility for rare, valuable or difficult to collect wild aquatic animals, where direct sampling of animals is undesirable or cost prohibitive. They may also provide cost advantages for disease monitoring programs in production environments.

3. There are currently no frameworks to allow evaluation of diagnostic performance of eDNA methods in a manner similar to animal-derived samples. For this reason, evidence from eDNA detection methods cannot be used as evidence for self-declaration of freedom from disease.

4. eDNA methods will be considered for inclusion in disease-specific chapters of the *Aquatic Manual*, if minimum disease and reporting standards as described in this paper are met.

5. Positive results from an eDNA method that has been included in the *Aquatic Manual* will be considered as an appropriate criterion for a suspect case of a disease.

6. The application of eDNA methods for a given purpose should be considered carefully with respect to the pathogen to be detected, the environment to be sampled, the reliability of the method and the implications of positive results that may require surveys of susceptible animal populations to confirm or exclude a suspect case.

7. Positive results from an eDNA methods that has been included in the *Aquatic Manual* will <u>not</u> be considered as an appropriate criterion for a confirmed case of a disease in either apparently healthy or clinically affected animals.

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Appendix 1. Publications describing eDNA methods for aquatic animal pathogenic agents

Table 1. Published applications of eDNA methods for the detection of
WOAH listed pathogenic agents of aquatic animals

WOAH listed disease	Publication
Amphibian diseases	
Infection with *Batrachochytrium dendrobatidis*	Brannelly *et al.*, 2020; Julian *et al.*, 2019; Kamoroff & Goldberg, 2017; Mosher *et al.*, 2017; Pierson & Horner, 2016; Walker *et al.*, 2007
Infection with *Batrachochytrium salamandrivorans*	Brunner, 2020; Spitzen-van der Sluijs *et al.*, 2020
Infection with Ranavirus species	Hall *et al.*, 2016; Julian *et al.*, 2019; Miaud *et al.*, 2019; Pierson & Horner, 2016; Vilaca *et al.*, 2020
Fish diseases	
Infection with *Gyrodactylus salaris*	Fossoy *et al.*, 2020; Rusch *et al.*, 2018;
Infection with HPR-deleted or HPRO infectious salmon anaemia virus	Gregory *et al.*, 2009
Infection with koi herpesvirus	Haramoto *et al.*, 2007; Honjo *et al.*, 2010; 2012
Infection with salmonid alphavirus	Bernhardt *et al.*, 2020; Weli *et al.*, 2021
Crustacean diseases	
Acute hepatopancreatic necrosis disease	Kongrueng *et al.*, 2015
Infection with *Aphanomyces astaci* (crayfish plague)	Robinson *et al.*, 2018; Rusch *et al*, 2020; Strand *et al.*, 2011; 2014; Vralstad *et al.*, 2016; Wittwer *et al.*, 2018a; 2018b
Infection with white spot syndrome virus	Natividad *et al.*, 2008; Quang *et al.*, 2009
Mollusc diseases	
Infection with *Bonamia ostreae*	Jorgensen *et al.*, 2020
Infection with *Perkinsus marinus*	Audemard *et al.*, 2004
Infection with *Xenohaliotis californiensis*	Lafferty & Ben-Horin, 2013

Table 2. Published eDNA studies of pathogenic agents of aquatic animals not listed by WOAH

Subject	Publication
Ornamental fish parasite detection	Trujillo-Gonzalez *et al.*, 2019b; 2019a
Parasitology	Bass *et al.*, 2015
Protozoan parasite outbreaks in fish farms	Bastos Gomes *et al.* 2017; 2019
Disease transmission in open water Salmon cages	Salama & Rabe, 2013
Emerging aquatic parasites	Sana *et al.*, 2018
Pathogenic microbes in bait	Mahon *et al.*, 2018
Waterborne virus detection	Oidtmann *et al.*, 2018
Halioticida noduliformans in lobsters	Holt *et al.*, 2018
Microcytos mackini	Polinski *et al.*, 2017
Trematode parasite *Ribieroia ondatrae*	Huver *et al.*, 2015
Schistosoma species	Alzaylaee *et al.*, 2020

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