SURVEILLANCE FOR INFECTION WITH *BONAMIA OSTREAE*

1. Purposes of aquatic animal health surveillance

Surveillance is the systematic on-going collection, collation and analysis of information related to animal health and the timely dissemination of information for policy and control actions. Although more details for developing surveillance systems are provided in the OIE *Guide for Aquatic Animal Health Surveillance* and in Chapter 1.4 *Aquatic animal health surveillance* of the *Aquatic Animal Health Code*, this document applies these concepts to a specific disease of molluscs: infection with *Bonamia ostreae*.

Surveillance for infection with *Bonamia ostreae* can be used to provide data on which claims of disease freedom can be based. It can also be used to describe disease occurrence, and for early detection of emergence of the pathogen in an area or of an outbreak in a population of cultured or wild oysters.

2. *Bonamia ostreae* transmission in the aquatic environment

2.1. Transmission mechanisms

The life cycle of *B. ostreae* is not completely known, but the disease can be directly transmitted between oysters in a population or experimentally by cohabitation or inoculation (Elston *et al.,* 1986; Hervio *et al.,* 1995), suggesting that an intermediate host is not required for the parasite to complete its life cycle. The observation of free parasites in gill epithelia, potentially associated with gill lesions, supports the hypothesis of a parasite release through these organs (Montes *et al.,* 1994).

Transmission of *B. ostreae* between oysters probably occurs through the water column. However, the infective form and routes of entry and release remain undetermined. Most *B. ostreae* might be released into the water column after oyster death through tissue lysis. A lag time of at least 3 months is generally observed before detecting the parasite in disease-free batches moved into endemic areas (Montes, 1991; Tigé *et al.,* 1980). Moreover, the infection seems to remain present in areas that have been depopulated and that have ceased to produce oysters for several years (Van Banning, 1988). The lag time before infection and persistence of the disease in depopulated areas has motivated some authors to investigate potential involvement of macro-invertebrate and zooplankton species in the *B. ostreae* life cycle (Lynch *et al.,* 2006). Nevertheless, considering the possible correlation between density of oysters and prevalence of bonamiosis (Grizel *et al.,* 1987), it seems that the parasite mainly depends on flat oysters (*Ostrea edulis*) for its survival and spread, and other aquatic organisms are probably not involved as important carriers or transmitters (Van Banning, 1988).

Considering the possible survival of *B. ostreae* in sea water for at least 1 week (Arzul *et al.,* 2009), any bivalve species able to maintain sea water inside its shell cavity can present a risk of transmission. Some recent results have suggested that *Crassostrea gigas* and *Pecten maximus* may act as vectors of *B. ostreae* (Lynch *et al.,* 2010).

All life stages of the oyster can be infected, including larvae (Arzul *et al.,* 2011), juveniles (Lallias *et al.,* 2008; Lynch *et al.,* 2005) and adults (Culloty & Mulcahy, 1996; Grizel, 1985). However, individuals older than 2 years appear to be more susceptible to the disease (Balouet *et al.,* 1983; Cullotty & Mulcahy, 1996; Engelsma *et al.,* 2010; Grizel, 1985; Robert *et al.,* 1991), and death is usually concurrent with the highest intensity infection level (Bréhelin *et al.,* 1982; Caceres-Martinez *et al.,* 1995; Montes *et al.,* 2003).

The flat oyster (*Ostrea edulis*) has an unusual feature: after female gamete fertilisation in the palleal cavity, the eggs and then the larvae are kept for an incubation period of 8–10 days before the larvae are released into the water. Infection in incubated larvae suggests that the parasite is transmitted during the incubation period from the mother to the larvae. Larvae may thus contribute to the dispersal of the parasite during its planktonic life according to the water currents.

Benthic macro-invertebrates and zooplankton species from a *B. ostreae*-endemic area were screened by polymerase chain reaction (PCR) for the presence of parasite DNA, and some of them yielded positive results. These species were used in laboratory transmission trials to investigate if they could infect naïve oysters. Transmission of *B. ostreae* was effected to two naïve oysters cohabiting with the brittle star, *Ophiothrix fragilis* (Lynch *et al.,* 2006).

Although the disease occurs and can be transmitted throughout the year (Tigé & Grizel, 1984), there is a seasonal variation in infection with *B. ostreae*. Prevalence of infection in an infected population presents peaks in late winter and in autumn (Arzul *et al.,* 2006; Culloty & Mulcahy 1996; Engelsma *et al.,* 2010; Grizel, 1985;

Montes 1990; Van Banning, 1991). Severely infected oysters die, mainly at the end of spring. Thus, if adult oysters are collected during the summer, prevalence in the population might be very low. In these cases low prevalence may lead to a lower sensitivity of detection, and therefore, apparent prevalence in the population may be even lower.

2.2. Pathogen transmission in aquaculture

Transmission of *B. ostreae* between oyster farms may occur through transfer of infected animals, or by other activities, for example, water transportation (e.g. boats, rafts, etc.). Transmission between populations in different sea beds has been documented in New Zealand for a closely related parasite, *B. exitiosa* (Cranfield *et al.,* 2005).

In addition, *B. ostreae* is able to survive in sea water for at least 1 week (Arzul *et al.,* 2009) and thus it could potentially be transmitted from an oyster farm to another through the currents, although such transmission has not been substantiated.

Infected larvae can contribute to the spread of the parasite, during the planktonic life stage of the oyster.

2.3. Transmission between farmed and wild aquatic animals

Transmission between farmed and wild aquatic animals is considered to follow the same mechanisms as in farmed populations. Clinical disease is observed in both farmed and wild populations.

2.4. Other transmission considerations

Water-borne transmission is probably important within a farm or a population, but might be less important between farms compared with transmission by larvae or by moving infected animals. *Bonamia ostreae* is able to survive in sea water for at least 1 week and thus can be transmitted from an oyster farm to another through currents. There are no data available concerning the presence and survival of *B. ostreae* in sediment and organic matter.

3. Populations

3.1. The concept of population applied to surveillance of infection with *Bonamia ostreae*

3.1.1. Population structure

A population can include several age stages:

i) larvae, which can be infected during the incubation period in the parent animals (spawners) and which then can spread the parasite;

ii) spat: after metamorphosis, larvae settle on supports and will not move by themselves (transfers by humans only); transmission can occur from infected animals to healthy ones. Infected animals will usually appear healthy, but mortality at this stage has already been observed under experimental conditions;

iii) adults, which will be moved for growing or marketing purposes, are considered as more infected (prevalence and infection levels higher) and more affected (more mortality)

In addition, based on the origin of the oysters, a population might include oysters produced in hatcheries and oysters produced naturally (in the wild).

It is possible to produce populations of resistant strains through selection programmes (Culloty *et al.,* 2004; Naciri-Graven *et al.,* 1998). Such oysters have been shown to perform better than non-selected oyster populations in terms of prevalence and intensity of infection, and to have lower mortalities.

3.1.2. Susceptible species

Natural host: European flat oyster is the only known natural susceptible species. In infected *O. edulis* populations, infection intensity (as well as mortality) increases with age and/or size of the oysters (Caceres-Martinez *et al.,* 1995; Culloty & Mulcahy, 1996; Grizel, 1985).

Oyster species that can be infected when moved into endemic zones: *Ostrea puelchana, O. angasi, O. chilensis* (= *Tiostrea chilensis, T. lutaria*) (Bougrier *et al.,* 1986; Bucke & Hepper, 1987; Grizel *et al.,* 1983; Pascual *et al.,* 1991).

Ostrea conchaphila (= *O. lurida*), *Crassostrea angulata* and *C. ariakensis* (= *C. rivularis*) have been speculated to be infected with *B. ostreae* (Cochennec *et al.,* 1998; Farley *et al.,* 1988; Katkansky *et al.,* 1969), but confirmatory diagnosis has not been achieved.

Results obtained on *Crassostrea gigas* suggested that this species can be infected with *B. ostreae* without any clinical signs. Subsequent exposure of naïve flat oysters to experimentally infected *C. gigas* led to the recovery of *B. ostreae* DNA from two of the flat oysters (Lynch *et al.,* 2010).

Flat oysters naïve to *B. ostreae* could be used as sentinels. However, they may not be useful for early detection because it may take from several weeks to several months following exposure to *B. ostreae* before sufficient prevalence and intensity is reached to allow detection in sentinel animals.

3.1.3. Disease exposure history

Oyster seed from natural settlements appears to be significantly more parasitised than oyster seed from hatcheries (Conchas *et al.,* 2003). Reduced mortality caused by bonamiosis could be achieved by reducing spat density, suggesting that increasing density might induce increased prevalence and infection intensity (Grizel *et al.,* 1987). However, some field studies did not demonstrate a direct correlation between density and prevalence of the disease (Engelsma *et al.,* 2010; Van Banning, 1991).

Stress related to transfer, dredging or handling of the oysters might contribute to development of the disease or to increased infection intensity.

3.1.4. Higher probability samples

It is more likely that *B. ostreae* will be detected in adult flat oysters from an infected area or in animals that have been temporarily immersed in an area infected with the parasite. Oysters that have endured stressful events, including spawning, dredging, handling, might be more susceptible to the infection and can be included in samples for risk-based surveillance.

Although many infected oysters appear normal, others may have yellow discoloration and/or extensive lesions (i.e. perforated ulcers) in the connective tissues of the gills, mantle and digestive gland, and such lesions could be suggestive of bonamiosis.

Following introduction of *B. ostreae* into a naïve population, high oyster mortalities can be expected for at least 6 years (Van Banning, 1991). Therefore, the observation of elevated mortality in adult flat oysters may indicate presence of the disease, although other conditions can induce increased mortality in flat oyster populations.

3.2. Host factors affecting population definitions

3.2.1. Carrier states

Infected oysters may have no clinical signs of disease, but can contribute to transmission of *B. ostreae*.

Natural resistance to bonamiosis has been suspected, and resistant strains against *B. ostreae* have been developed. These animals can be infected, but present lower prevalence and intensity of infection, and lower or later mortality (Bédier *et al.,* 2001; Culloty *et al.,* 2004; Elston *et al.,* 1987). Such animals could act as carriers of the parasite.

3.2.2. Factors affecting disease expression

Individuals older than 2 years appear to be more susceptible to the disease and, therefore, prevalence and mortality in such populations can be higher (Culloty & Mulcahy, 1996; Grizel, 1985). Both 0+ and 1+ year-old *O. edulis* are susceptible to infection, and high prevalence and intensity of infection can be observed in a newly infected population (Lynch *et al.,* 2005). Oyster seed from natural settlements appear to be significantly more parasitised than seed from hatcheries (Conchas *et al.,* 2003).

Stress related to spawning, transfer, dredging or handling of the oysters might contribute to the development of the disease. Prevalence has been shown to be higher at a low temperature (10°C) compared with a higher temperature (20°C), suggesting that low temperatures may affect defence capacities of the oyster and/or the ability of the parasite to infect healthy oysters (Cochennec & Auffret, 2002).

Some farming practices such as bottom or table culture, and co-cultivation with other species such as *C. gigas* seem to have an impact on the occurrence of the infection.

3.2.3. Group-level susceptibility

Naïve flat oysters are more susceptible than oysters originating from endemic areas/populations.

Natural populations seem to display different levels of natural resistance to bonamiosis (Elston *et al.,* 1987). Resistant strains that have been produced through selection programmes are less susceptible to the disease (Culloty *et al.,* 2004; Naciri-Graven *et al.,* 1998).

3.2.4. Vaccination

Not possible in molluscs.

3.3. Epidemiological units

3.3.1. Sharing risk of exposure

Oysters from the same natural bed or the same oyster park will have a similar level of risk of exposure.

Risk of exposure inside a geographical area (e.g. a bay or a lagoon) will depend on the water current dynamics and the distance between animals/farms/beds. In addition, environmental parameters including temperature and salinity influence the survival of the parasite in the sea water (Arzul *et al.,* 2009). In a given geographical area, proximity to a source of freshwater could influence risk exposure.

Epidemiological units can also be defined within a farm. For example, wherever ropes are used, a series of ropes or an individual rope can be considered an epidemiological unit.

3.4. Zones and compartments

Zoning is commonly used in the context of bonamiosis surveillance. Zones are defined on the basis of the hydrodynamic characteristics of the zone and the transfers of oysters. Oysters are sessile organisms and, therefore, they will be moved only if they are transferred or if the support on which they are fixed moves (e.g. boats). During their planktonic stage (=larvae) they can move by water currents. Thus if zoning is well designed, i.e. takes into account hydrodynamic characteristics, these natural movements should not disrupt zoning capability.

Compartmentalisation is possible for hatcheries, ponds, purification centres, and other semi-closed systems that treat water entering the facility (e.g. filtration at 1 µm) or use another source of seawater that is free from the parasite and infected animals.

3.4.1. Geographical barriers

Bays and harbours are usually well defined and easily identifiable. For a long coastline, the distribution of oyster farms/oyster beds should be taken into account: a coast line without susceptible animals could separate two zones.

Bonamia ostreae is transmitted from infected oysters to healthy ones by cohabitation, suggesting that close contact favours parasite transmission. However, larvae can also become infected and thus can contribute to the spread of the parasite during its planktonic stage. *Bonamia ostreae* is able to survive for at least 1 week in sea water and can, thus, be transmitted from an oyster farm/bed to another by currents. Oysters fixed on boats are suspected to be the route of transmission for *B. ostreae* to some previously free areas (Howard, 1994).

Natural epidemiological separation of populations may occur if there are some geographical barriers (e.g. limit of a bay or harbour), hydrodynamic barriers and/or intermediate areas without susceptible animals.

3.4.2. Prevalence zones

In an area/zone where infection is endemic, most farms are expected to have infected animals. Within an infected population in a farm or natural bed, prevalence can be very variable (from 1% to 80%). The disease occurs and can be transmitted throughout the year, but there is a seasonal variation in infection with *B. ostreae*. The maturation and spawning period (i.e. summertime) could constitute a period of weakness during which the oysters are less able to resist infection by the parasite and will

die. This can result in increased transmission leading to the observation of peaks in prevalence of infection later in the year (Arzul *et al.,* 2006; Culloty & Mulcahy, 1996; Grizel, 1985; Montes, 1990; Van Banning, 1991).

4. General design considerations

4.1. Types of surveillance system

4.1.1. Passive surveillance

Passive surveillance for *B. ostreae* will involve observation of clinical signs or increased mortality of oysters followed by reporting of these observations, sample collection and laboratory testing. Oysters infected with *B. ostreae* often appear normal but others may have yellow discolouration and/or extensive lesions (i.e. perforated ulcers) in the connective tissues of the gills, mantle and digestive gland. Mortality of flat oysters or the presence of clinical signs consistent with bonamiosis may be recognised by oyster farmers, fishermen, transporters or any other person in contact with the oysters. In the case of a suspected outbreak, sampling of affected oyster populations and laboratory testing for *B. ostreae* should be performed to confirm or exclude it as a cause.

Samples that have been submitted to a laboratory for reasons other than investigations for bonamiosis can also be checked for the presence of *B. ostreae* and thus be incorporated into passive surveillance systems.

Cultural practices in flat oyster rearing in some countries (on the seafloor bottom in deep water) mean that farmers or fishermen may not see their oysters every day and they may observe and report increased mortality quite late after an outbreak. Furthermore, because of the lack of specific clinical signs and the fact that infection will not consistently lead to death of the animals in a short period of time, passive surveillance alone may not be sufficient for some purposes (e.g. early detection) and may need to be used in combination with active surveillance.

4.1.2. Active surveillance

Active surveillance for *B. ostreae* will involve a structured program of health inspections, oyster sampling and laboratory testing. The design of the program will depend on its specific purpose; for example, early detection, to support claims of disease freedom, or to describe the nature of the disease within a population.

It is recommended that active surveillance be combined with passive surveillance for the declaration and maintenance of disease free status. Regular health inspections may be planned and oyster sampling should be designed to be representative of the population based on its structure.

Where the objective of active surveillance is early detection of the pathogen, risk-based surveillance could be undertaken by sampling epidemiological units with a higher risk of introduction and spread of *B. ostreae*. These may include hatcheries, areas with high boat traffic, areas with higher risk animal movement, and purification centres.

If the objective of active surveillance is the estimation of prevalence of disease or infection, careful consideration of the sampling design is required to ensure it is representative of the population under study.

4.2. Sampling from markets or processing plants

Sampling at markets can be undertaken for identifying infected individuals and, therefore, revealing that infection is present in a population. However, it might be difficult to determine the exact origin of the sampled animals. Considering the frequency and importance of transfers in oyster production, sampling oysters from markets or processing plants would not allow reliable determination of country/zone/compartment status. Sampling from markets may have some utility in circumstances where the origin of oysters is known with certainty.

4.3. Questionnaire-based surveillance

Questionnaires could be directed to farmers, animal health authorities, veterinarians or to the people involved in marketing. This might produce useful information concerning the presence of the infection and it can direct sampling to populations at higher risk. However, because of the lack of specific clinical signs and the fact that many infected oysters appear normal, it is possible that questionnaire-based surveillance could have low

sensitivity. A surveillance system combining questionnaire administration and active sampling may produce information that satisfies the surveillance objectives.

4.4. Case definition

The OIE defines the disease infection with *Bonamia ostreae* as *infection* with the haplosporidian parasite *B. ostreae*. Oyster mortality or presence of clinical signs consistent with bonamiosis may be incorporated into a surveillance program; however, it is necessary to confirm the presence of *B. ostreae*.

The OIE Aquatic Manual (2013) defines a suspect case of infection with *B. ostreae* as a positive result obtained with any of the diagnostic techniques described in the infection with *B. ostreae* chapter of the OIE Aquatic Manual.

A confirmed case of infection with *B. ostreae* is:

- In susceptible species within the known geographical range of infection with *B. ostreae*, a positive result by tissue imprints, histology or in-situ hybridisation combined with a positive result by PCR-RFLP and sequencing or SYBR Green real time PCR.
- In other host species or outside the known range of *B. ostreae*, TEM confirmation is recommended.

4.5. Surveillance and denominator-based information

Reservoirs of *B. ostreae* include carriers among cultured and wild flat oysters. Several factors influence susceptibility to bonamiosis. Flat oysters may display genetic variability for susceptibility (Elston *et al.,* 1987), and the age of the oysters appears to be of some importance—the older oysters are usually more infected and more likely to die of bonamiosis than young ones. The population at risk, especially in naïve oysters, would thus be all sizes and life stages. Whole farm stocks or population of wild susceptible oysters should therefore be considered at risk.

5. Diagnostic tests

The following table summarises available information on sensitivity and specificity of some tests for detection of *B. ostreae*, from published and unpublished studies. The estimations reported in the table were obtained on oysters collected from the field.

Table 5.1. *Summary of available information on sensitivity and specificity of some tests for detection of* B. ostreae

	Histology	**Heart imprints**	**PCR (Cochennec *et al.,* 2000)**	**qPCR (Robert *et al.* 2009)**	***In-situ* hybridisation (Cochennec *et al.,* 2000)**
Diagnostic Se	0.66 (relative to PCR) 0.85 (relative to imprints)**	0.4*	0.84*	No data	No data
Diagnostic Sp	0.98 (relative to PCR) 0.88 (relative to imprints)**	1*	1*	No data	No data
Analytical Se	low	low	at least tenfold less sensitive than qPCR	50 copies/PCR reaction at least tenfold more sensitive than conventional PCR	Higher than histology
Analytical Sp	Genus level	Genus level	Genus level + some *Haplosporidium* species	Species level	Genus level + some *Haplosporidium* species
Repeatability				Variation <1%	

Table 5.1. *Summary of available information on sensitivity and specificity of some tests for detection of* B. ostreae

	Histology	**Heart imprints**	**PCR (Cochennec *et al.,* 2000)**	**qPCR (Robert *et al.* 2009)**	***In-situ* hybridisation (Cochennec *et al.,* 2000)**
Reproducibility	Based on three inter-laboratory comparison tests including 19–20 laboratories*** Mean of good responses is 77.96% More discrepancy for light infections	Based on three inter-laboratory comparison tests including 19–20 laboratories*** Mean of good responses is 81.42% More discrepancy for light infections	Based on 1 inter laboratory comparison test including 17 laboratories*** mean of good responses is 89.54% More discrepancy for light infections	No data	

PCR = polymerase chain reaction; qPCR = real-time PCR; Se = sensitivity; Sp = specificity.
*Maximum likelihood estimation method (TAGS: Pouillot *et al.*, 2002) using haemagglutination inhibition and PCR data on three populations of oysters: negative (90 oysters), lightly infected (200 oysters) and moderately infected (200 oysters) unpublished data (Arzul, pers. comm.). **Estimates were calculated using data published by Balseiro *et al.,* 2006: comparison of histology, PCR and examination of gill imprints from 240 oysters (eight samples of 30 oysters). ***Data from the EURL for mollusc diseases (2010)

Table 5.2. *Some information and empirical assessments concerning available tests*

	Histology	**Heart imprints**	**PCR (Cochennec *et al.,* 2000)**	**qPCR (Robert *et al.,* 2009)**	***In-situ* hybridisation (Cochennec *et al.,* 2000)**
Advantages	Good screening method as it allows examination of all the organs Allows detection of other pathogens (including the listed protozoan *Marteilia refringens*)	Easy screening method and interesting presumptive method because it is inexpensive, very quick and easier than histology to evaluate	Less specialised knowledge is required Easy screening method	Less specialised knowledge is required Easy screening method More sensitive than conventional PCR Species specific Allows quantification of the parasite	Less specialised knowledge is required Allows detection of low levels of infection
Disadvantages	Requires specialised knowledge and experience Difficulty to detect low level of infection Does not allow parasite species determination	Requires specialised knowledge and experience Difficulty to detect low level of infection Only considers a small piece of the animal: heart (or gills) Does not allow parasite species determination	Difficulty to detect low level of infection Only a small piece of gill tissue from the animal is tested Does not allow parasite species determination (needs to be combined with RFLP/sequencing for species characterisation)	Only considers a small piece of gills from the animal	Not applicable for routine screening Does not allow parasite species determination

PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism.

5.1. Assessment of consistency across multiple laboratories that would be involved in the surveillance

Inter-laboratory tests may be necessary to assess consistency of test results among different laboratories, particularly where multiple laboratories are performing testing for a surveillance program. They should be organised at regular intervals by laboratories accredited to coordinate proficiency testing.

Inter-laboratory tests are particularly important for histological and cytological testing because discrepancies may occur due to different levels of experience of laboratory staff, or due to different operating procedures.

Two potential versions of inter-laboratory tests are presented below:

i) A collection of samples of known infection status is sent to all laboratories and agreement of individual sample results is assessed. This is common practice among European Reference laboratories.

ii) Several collections of samples of unknown infection status and prevalence are sent to all laboratories and agreement of prevalence results is assessed.

Generally, maximum discrepancies are observed for lightly infected samples. For example, for the histocytology test, an average of 80% of correct results (on 30 slides) can be considered acceptable while an average of between 60 and 80% of correct test results indicate that the laboratories results should be taken with caution. Less than 60% of correct test results is unacceptable and indicates that additional training is required.

For PCR-based tests, one error or no error (on 24 tested samples) is perfect, errors only on samples yielding maximum discrepancies among laboratories is considered good while errors in addition to or instead of the previous ones should be investigated to find out the source of the problem.

5.2. Expected effect of incorrect or inadequate storage/handling of samples after collection

Histology and cytology (imprints) can only be performed on live or gaping (freshly dead) oysters. These techniques are not applicable if tissues are degraded.

For PCR testing, analytical sensitivity will probably decrease if tissues are not well preserved due to DNA degradation.

5.3. Effect of pooling

Some tests have been carried out to compare PCR results on individual oysters, pools of three and pools of five oysters. Pooling induced a decrease of the PCR sensitivity (unpublished results). Pooling of samples is not recommended unless the impact on diagnostic test performance has been evaluated.

6. Sampling considerations for surveillance

6.1. General considerations

Sampling of aquatic animals is more relevant to active surveillance, but can also be used in passive surveillance to facilitate outbreak investigations or other instances of increased disease occurrence.

Sampling design depends on the objective and scope of the study and on the structure of the examined populations. There is a major distinction between studies that aim at estimating the occurrence of infection in a population and studies that aim at detecting infection or claiming that the population is free from the infection. In the first case, a representative sample is sought, while in the second case, sampling may be biased towards detection of the infection or the disease, i.e. animals with signs or moribund animals could be selected, but often few such animals can be found in a naturally infected population. Sampling can also be targeted at high-risk groups within a population, for example adult animals, especially after spawning.

The number of units to be sampled from a population should be calculated using a statistically valid technique that takes at least the following factors into account:

- The sensitivity and specificity of the diagnostic test or test system;
- The desired minimum detectable prevalence (or prevalences where a multi-stage design is used) or design prevalence;
- The sensitivity of the system (for supporting claims of disease freedom);

- The precision desired (i.e. the width of the confidence or probability intervals).

Additionally, other factors may be considered in sample size calculations, including (but not limited to):

- The size of the population (but it is often acceptable to assume that the population is infinitely large);
- The desired power of the survey;
- Uncertainty about sensitivity and specificity.

Natural variations in disease occurrence throughout the year should be considered when designing the surveys. For example, as it has been observed that infection prevalence naturally peaks in late winter and in autumn, different surveys may be required at different times of the year to estimate the prevalence of infection at those times. On the other hand, if detection of infection is the objective, then the sensitivity of the detection system can be increased by conducting the survey at the times of the year when prevalence in infected populations peaks.

In an area with a low level of prevalence (e.g. 2%), mortality is usually not reported. It is not possible to determine a minimum expected prevalence for this infection.

6.2. Sample sizes

The number of individuals to be sampled in a surveillance programme is a major determinant of the probability of detecting diseased individuals from the population. If the sample size is too small, surveillance might well fail to detect diseased individuals, even if these exist at relatively high prevalence. On the other hand, a very large sample size will detect a smaller prevalence of disease but may be costly and inefficient.

Choosing a suitable sample size depends on statistical considerations, such as the desired precision and expected disease frequency, and non-statistical considerations, such as resources, cost and sample availability. The sample size affects the precision of the estimated prevalence. In addition, the expected frequency of disease in the population will affect the estimate.

The structure of the population needs to be considered in the design of the survey and the calculation of sample sizes. For example, the survey may concern only one farm or site, or it may be targeted at multiple sites or an entire region or country. In the latter cases, sampling should be conducted separately at each stage. For example, a sample of regions could be taken from all the regions in the country (sampling unit: the region), next a sample of farms within each selected region (sampling unit: the farm), next a sample of ropes within each farm (sampling unit: the rope) and finally a sample of animals from each selected rope (sampling unit: the animal). Special attention should be given to the definition of design parameters (e.g. design prevalence, test sensitivity and specificity) at each sampling level. For example, the region-level prevalence would be the proportion of regions in the country that are infected, while the farm-level prevalence would be the proportion of infected farms in a region, etc. For more details, examples and calculations, the reader is directed to chapter 1.4 of the OIE *Aquatic Animal Health Code* and to the OIE *Guide for Aquatic Animal Health Surveillance*. In the case of *B. ostreae,* a collection of units (i.e. the farm) will be classified as infected if at least one of its units is found to be infected. In this case, test sensitivity and specificity should be defined accordingly at the farm level (or any other collection-of-units level).

A few example calculations for sampling from a single population level are presented here.

6.2.1. Specification of design prevalence values

The values for design prevalence used in calculations for sample size or analysis of data from surveys for disease freedom must be those specified in the relevant disease chapter of the *Aquatic Manual*. In the case of *B. ostreae* such values are not specified in the *Aquatic Manual*. Therefore, a suitable value of design prevalence should be selected and this selection should be justified. Some issues that need to be considered are outlined here.

6.2.1.1. Dynamics of infection

At the individual aquatic animal level, the design prevalence is based on the biology of the infection in the population. It is equal to the minimum expected prevalence of infection in the study population, if the infection has become established in that population. Design prevalence is dependent on the dynamics of infection in the population and the definition of the study population. If a minimum expected prevalence cannot be specified, the design prevalence will be the maximum acceptable prevalence, i.e. the prevalence detection limit of the surveillance system.

6.2.1.2. Design prevalence value

A suitable design prevalence value at the aquatic animal level (e.g. prevalence of infected aquatic animals in an oyster bed) may be:

- Between 1% and 5% for infections that are present in a small part of the population, such as those that are transmitted slowly or are at the early stages of an outbreak;
- Over 5% for highly transmissible infections.

If reliable information on the expected prevalence in an infected population is not available, a value of 2% should be used for the design prevalence.

Bonamiosis prevalence may fluctuate depending on the infected populations:

- Some infected populations present low prevalence (below 2%) where mortality is usually not observed;
- Some populations infected for more than 20 years present a moderate to high prevalence fluctuating between 2 and 35% depending on the year and the season (Arzul *et al.,* 2006; Englesma *et al.,* 2010);
- In outbreak situations, prevalence can be even higher.

6.2.1.3. Complex population structure

In the presence of a complex (e.g. multi-level) population structure, more than one design prevalence value is required. For instance, the values might refer to the aquatic animal-level prevalence (proportion of infected aquatic animals in an infected farm) and the group-level prevalence (proportion of infected farms in the country, zone or compartment). In these cases, it is necessary to define the number of units in the cluster that need to test positive for the cluster to be considered infected. The proportion of truly infected clusters (i.e. farms) that the cluster-level test identifies as positive is the cluster-level sensitivity, while the proportion of truly uninfected clusters that the test identifies as negative is the cluster-level specificity. Further levels of clustering may be considered, requiring further design prevalence values, and sensitivity and specificity estimates, for testing at each level.

Some indicative calculations are included in the following table, using two different diagnostic tests (detailed characteristics reported in section 5). For prevalence estimation, sample sizes are provided for different levels of required precision (half of the width of the confidence interval) and assumed true population prevalence (0.02, 0.05, 0.10). Calculations presented are for an infinite population and confidence level of 0.95. For these calculations the online EpiTools epidemiological calculators were used (http://epitools.ausvet.com.au/content.php?page=home). For surveys for demonstrating disease freedom three different design prevalences were used (0.02, 0.05 and 0.10) and the probability of type I and type II error were set at 0.05 and a population size of 100,000. No cut-point number of reactors is presented as both tests are assumed to have a specificity of 1, hence no false positives are expected. Calculations were done using the FreeCalc software (Survey Tool Box: AusVet Animal Health Services. Includes FreeCalc – Cameron AR Software for the calculation of sample size and analysis of surveys to demonstrate freedom from disease. Available for free download at: http://www.ausvet.com.au/content.php?page=software).

Objective	Required precision	Assumed true population prevalence	Heart imprints (Se: 0.4; Sp: 1)	PCR (Se: 0.8; Sp: 1)
Prevalence estimation	0.05	0.02	77	38
	0.02		477	237
	0.01		1906	945
	0.05	0.05	189	93
	0.02		1177	577
	0.01		4706	2305

Objective	Required precision	Assumed true population prevalence	Heart imprints (Se: 0.4; Sp: 1)	PCR (Se: 0.8; Sp: 1)
	0.05	0.10	369	177
	0.02		2305	1105
	0.01		9220	4418
Disease freedom demonstration		0.02*	373	186
		0.05*	149	74
		0.10*	74	36

PCR = polymerase chain reaction; Se = sensitivity; Sp = specificity
*design prevalence

6.3. Sampling units

6.3.1. Individual to aggregate

The sample unit depends on the stage of sampling. For example, sampling units can be (in ascending stage order): oyster, bag/rope, trestle/line, park/bed, farm, area.

6.4. Sampling strategies

6.7.1. Sampling in disease situations

Sometimes, infected oysters have yellow discoloration and/or extensive lesions (i.e. perforated ulcers) in the connective tissues of the gills, mantle and digestive gland. However these lesions are not pathognomonic and most of the oysters will be asymptomatic. Furthermore, a gaping oyster that is able to close its valves again will be considered moribund. If the oyster is gaping and not able to close its valves anymore, it is dead. When the objective of the survey is detection of infection, moribund oysters should be sampled preferentially, since they are more likely to have more severe infections than live ones. It should be noted that histology and cytology (imprints) can only be performed on live or gaping (freshly dead) oysters. These techniques are not applicable if tissues are degraded. Analytical sensitivity will probably also decrease for PCR when tissues are not well preserved because of DNA degradation. Priority can also be given to freshly dead specimens (especially at the end of spring-beginning of summertime). By sampling these oysters, one can expect to increase sensitivity of detection.

6.5. Probability sampling

Effective probability sampling requires the existence of good records of the holding units in which the sampled populations are organised. Information on location, size and other characteristics of the holding units needs to be available. Surveys are most successful in areas where all the holding units are registered. Sampling frames (lists of all units) are generally needed, but other approaches that do not require the existence of a sampling frame (e.g. systematic sampling) can be applied to certain levels of organisation of the population. Sampling frames of farms and of natural beds will usually be available. Within a farm, it is possible to produce a sampling frame of ropes, but not of individual animals. Probability sampling may also require maps of oyster farms and harvested beds and also maps indicating the distribution of the oysters in the bed, or the location of the bags, trays or ropes in the farm. Moreover, probability sampling usually requires more human time to collect oysters. Probability sampling may be more complicated than non-probability sampling but it is certainly possible and it is the recommended way to produce unbiased parameter estimates.

6.5.1. Practical in-field situations

Simple random sampling, as described in example 3 in Chapter 1.4, Article 1.4.11 of the *Aquatic Animal Health Code*, may present practical difficulties because one would be trying to locate and sample individual oysters, spread out in many different points according to the randomly selected coordinates. Therefore, other approaches, like cluster sampling (in which one would take a sample of clusters, e.g. ropes, and then sample every oyster on the selected ropes) might be more practical in some situations.

Describe whether it is possible to collect a true probability sample of animals:

This is possible. Several different methodologies can be used in different situations. Two possible examples are listed below:

1. Country>farms>lines of ropes>ropes>oysters

   One approach is described in example 3 in Article 1.4.11 of the *Aquatic Animal Health Code*. However, if not feasible, other sampling schemes can be designed. For example, in each farm, lines of ropes can be selected randomly (instead of individual ropes). This can be done either with simple random sampling or using geographical coordinates or by systematic sampling. Once the lines of ropes have been selected then individual ropes can be sampled and oysters from the selected ropes can be selected using similar approaches.

   The size of the farm must be taken into consideration, either for the probability of the farm being selected or for the number of animals sampled from each farm.

2. Country>natural oyster beds

   A random sample of natural beds in the country can be selected and then, within each natural bed, all individuals can be sampled by dredging or a random sample can be taken, either by dredging or by using geographical coordinate sampling, or by specifying a grid in the area and then selecting randomly a number of cells, either by simple random sampling or by systematic sampling. Inside each square, oysters will be collected randomly or subsquares will be drawn, if necessary; it may also be possible to sample all the oysters in the cell.

6.5.2. Systematic random sampling

Systematic random sampling is another method of probability sampling. A description of this methodology is given in Chapter 1.4, Article 1.4.11 of the *Aquatic Animal Health Code*. It may be possible to use systematic random sampling at several level of organisation of the targeted population or during other activities related to harvesting, transportation and marketing.

6.5.3. Potential biases introduced with systematic random sampling

Biased samples can be obtained if characteristics exist that may influence the risk of infection and that have a cyclical distribution and the frequency of systematic sampling is the same as the frequency of this cyclical distribution. For example, if every 10 ropes are sampled and ropes are arranged in lines of 10 then the sample will include only ropes from one specific side of the farm. If conditions affecting infection on that side of the farm are different or there are different risk factors for the infection or the disease compared with other areas of the farm, then samples obtained will probably be biased.

6.5.4. Probability sampling at different life/production stages

It is more difficult to obtain a probability sample when targeting larval stages. This can be done by sampling larvae directly from the water column. However, the sample might be more representative by collecting adults incubating their larvae.

6.5.5. Possibility of non-probability sampling for detection of *Bonamia ostreae*

If the objective is to assess disease freedom then sampling can be biased towards pathogen detection. In this case higher-risk groups (for example, individuals older than 2 years and/or moribund individuals) should be sampled preferentially. However, if the objective of the system is to estimate prevalence or describe disease occurrence, then non-probability sampling should be avoided.

6.5.6. Convenience and purposive samples

Oysters can be sampled in tanks where they are stored before human consumption, in markets, in purification centres, in transport means or from boats after harvesting. Oysters collected for public health surveillance purposes can also be used as convenience samples. In such cases, however, the representativeness of the samples will need to be addressed. Moreover, it may not be possible to determine the geographical area of origin of these samples. These are serious problems that may hamper the interpretation and limit the usefulness of the survey results.

6.5.7. Potential biases associated with using convenience sampling

As the distribution of infection in a population in a given area may not be homogeneous, considerable biases can be introduced using convenience sampling. The direction of the bias will depend on the criteria that lead to selection of specific animals and will be influenced by the specific characteristics of the animals that are included in the sample in relation to those that are not.

Examples of additional biases may include sampling high mortality areas, which could induce biases in the prevalence estimates obtained. In fact, underestimation may result from sampling after the peak of mortality while overestimation may occur if the prevalence is very high in the sampled stock and not representative of the target population. Similar biases may be produced when sampling is not done throughout the year and the seasonal fluctuation of the infection prevalence is not considered. Finally, sampling only harvested animals can induce an overestimation of the prevalence as marketed animals are more likely to be infected than younger animals. On the other hand, this strategy may, under several circumstances, produce an underestimation of the prevalence, if infection is highly lethal, and therefore animals that have survived up to the time of harvesting may have a higher probability of being non-infected.

6.5.9. Potential attributes that can be considered when adopting purposive sampling

Purposive sampling can be based on clinical signs, the size of the oyster, the time of the year, or the level of mortality of a part of the population.

6.5.10. Most common haphazard sampling methods and associated biases

The most common haphazard sampling methods are dredging, diving, choosing a bag/tray or a rope and collecting the required number of oysters.

Prevalence can be underestimated or overestimated. As data concerning the distribution of the infection in a farm/bed will not usually be available, it is not really possible to predict the direction of the bias.

6.6. Selection bias

6.6.1. Independence between individuals

Individuals held in the same holding units and having the same history will have the same chance of being infected. This may be different for individuals in other units. This fact should be considered in the sampling design and the statistical analysis of the results. Some factors that may affect infection and severity of disease are the age and size of the animals and the origin of the animals (geographical origin and hatcheries versus wild origin). Other factors such as cultural practices (bottom culture versus/table culture), and co-cultivation with other species such as *C. gigas* seem to have an impact on the occurrence of the infection. For more information see Section 3 above.

6.7. Multi-stage sampling

6.7.1. Groupings of characteristics or disease patterns

In the case of bottom culture, the bed or farm can be gridded and squares can be chosen randomly. Inside each square, oysters will be collected randomly or subsquares will be drawn, if necessary. In the case of oysters in bags (on tables organised in band) or trays (along strings), the first stage is the band or string and the second stage is the bag or tray and the third stage is the oyster.

In the case of oysters scotched on ropes, the first sample stage is the string/raft, and then the rope and then the depth and finally the oyster.

For more details, see Sections 6.5 Sample sizes and Section 6.8 Probability sampling.

7. Statistical aspects

7.1. Expertise required

In general, for simple surveillance systems in homogenous populations, analytical results can be obtained relatively easily by using simple software, which is often available as freeware. In more complex situations, and in populations with complex organisational structures, analytical methods should be thoroughly considered and

consultation with a biostatistician/quantitative epidemiologist is usually advisable, both at the planning stages and throughout the programme.

7.2. Quantifying uncertainty

In virtually any surveillance system, it will be impossible to test all aquatic animals that belong to the relevant population. Sampling is essential, but this always leads to a degree of uncertainty about the results. Statistical analysis of the data that result from the surveillance system aims at quantifying this uncertainty and aiding the decision process, which will necessarily be based on seeing a part of the overall population picture.

7.3. Statistical inference

7.3.1. Hypothesis testing and estimation

Statistical inference encompasses two approaches: hypothesis testing and estimation. The two use different procedures, but their mathematical background is common and their results are equivalent. Hypothesis testing is often used to analyse the results of surveys for disease freedom. In these cases, data are used to test the null hypothesis that the population is not free from disease. In surveillance to describe disease occurrence, the data are mostly used to estimate a measure of disease frequency in the target population or subpopulations defined by geographical or aquatic animal characteristics. There can be a single survey or a series of surveys. In surveillance for detection of new diseases, results are usually qualitative and no statistical analysis is necessary.

7.3.2. Confidence interval

Estimation is the statistical process of producing estimates concerning population parameters of interest, based on the data at hand. In other words, the sample results are used to produce some estimates about the value of the parameter of interest in the population from which the sample is obtained, and of which it is taken to be representative. The estimates are usually in the form of a confidence interval with a specified level of confidence (e.g. 95%) that the range will include the true value of the population parameter. The idea of confidence can only be interpreted in relation to repeated sampling. For example, 95% confidence means that if the same study was carried out 100 times (with a different sample from the same population) and each time a 95% confidence interval was calculated, then 95 of those 100 intervals would contain the true population parameter value, while five would not. It is possible to obtain an interval that will contain the true population value with higher confidence, but it will by definition contain a wider range of values. It is not possible to obtain a 100% confidence interval unless the entire population is tested.

7.3.3. Precision of the estimate

The width of the confidence interval expresses the precision of the estimate of the population parameter (e.g. prevalence). A narrow confidence interval indicates high precision. Such an interval is desirable because it contains a lot of information about the likely value of the population parameter. However, higher precision (a narrower confidence interval) is usually obtained by sampling and testing more animals. At the beginning of a study or a survey, it is essential to specify the amount of precision desired in the results, and calculate the number of animals that need to be tested to obtain this precision.

7.4. Sensitivity and power of the surveillance system

7.4.1. Sensitivity of the surveillance system

Analysis of data for evidence of freedom from infection involves estimating the probability (p) that the results of the surveillance could have been produced under the null hypothesis that infection is present in the population at a specified (or higher) prevalence (the design prevalence). The sensitivity of the surveillance system that produced the evidence is equal to ($1 - p$). This is the probability that the system would detect infection if infection were present at the specified design prevalence level. If ($1 - p$) exceeds a desired threshold (usually but not necessarily 0.95), the evidence is deemed adequate to claim freedom from disease (at the design prevalence level). Different statistical methodologies for the calculation of the probability ($1 - p$), including both quantitative and qualitative approaches, are acceptable provided they are based on accepted scientific principles. The methodology used to calculate ($1 - p$) must be scientifically based and clearly documented, including references to published work describing the methodology.

7.4.2. Power of the surveillance system

The power (the probability that the system will report that no infection is present if infection is truly not present) may be set to any value. By convention, this is often set to 80%, but it may be adjusted according to the country or zone requirements.

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* *

NB: There is an OIE Reference Laboratory for infection with *Bonamia ostreae*
(see Table at the end of this *Aquatic Manual* or consult the OIE Web site for the most up-to-date list:
http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, etc. for infection with *Bonamia ostreae*