Outbreak!
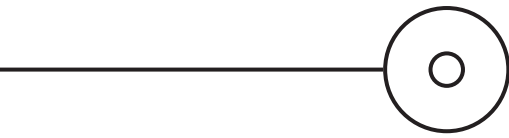
Investigation guidelines for
aquatic animal disease events

FRDC
FISHERIES RESEARCH AND
DEVELOPMENT CORPORATION

AGRICULTURE VICTORIA

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Cover photo. Salmon farm (Photo courtesy of Huon Aquaculture)

Contents

DEFINITION:

Outbreak

/ˈaʊtbreɪk/

When there is a higher number of animals affected by a disease[1] than is expected.

Photo. Fish kill in Western Australia (Photo courtesy of Mark Canny, DWER WA)

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1 Disease is defined as dead, unwell and dying aquatic animals irrespective of cause for these guidelines.

Introduction

These guidelines have been developed to use in the event of a disease outbreak in aquatic animals including finfish, molluscs and crustaceans. For the purposes of these guidelines, in the context of an outbreak, the term "disease" will be used for any event where there are animals that have clinical signs or are dead, irrespective of cause. The approach to determining what is affecting animals will be similar whether the cause is an infectious pathogen or an environmental issue such as a chemical toxin.

The outbreak investigation process follows 10 basic steps, but these steps do not necessarily need to be completed in sequence.

The information collected will provide the evidence required to conduct an epidemiological investigation. This will aid in understanding if the disease event is an outbreak, what may be causing the disease, what controls to apply and ideally, how to prevent future outbreaks.

Clear Information on diagnostic tests and how to submit the best sample possible is provided towards the end of the guidelines (see page 67).

Photo. Photoperiod lights over Chinook salmon raceways (Photo courtesy of Snobs Creek Hatchery)

Why do we need a guide on investigating disease outbreaks in aquatic animals?

Traditional perspective
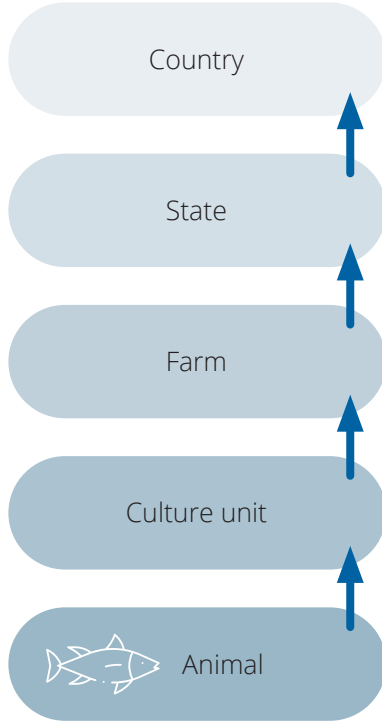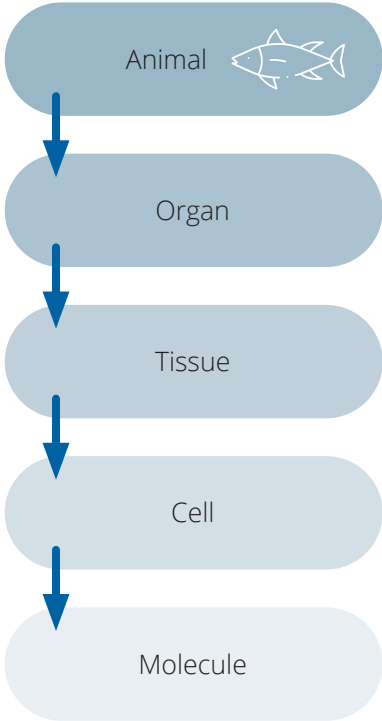
Population perspective

Figure 1. Representation of the relationship between the traditional perspective of investigating disease and a population perspective. Adapted from FRDC Project 2009–315 (2012)

Photo. Barramundi (Photo courtesy of Mainstream Aquaculture)

Epidemiological investigations into the causes of death and disease in a group of terrestrial animals have traditionally worked under the premise that **disease does not occur randomly**. In the sphere of aquatic animal health we are working in a complex ecosystem where it may be challenging to establish the underlying cause of a disease outbreak as the environment, underlying health condition of stock, management practices and the presence of infectious or toxic agents must all be considered.

The traditional approach in aquatic animal disease events is to concentrate on laboratory diagnoses in individual animals. When we look at the whole population, whether on a farm or in a wild environment, we aim to identify and analyse patterns of disease and ultimately institute control measures (Figure 1).

The logical "outbreak approach" as outlined in these guidelines has been followed for many years in terrestrial animals and should be more widely employed when investigating dead or diseased animals in the aquatic environment.

STEP 1

Confirm there is an outbreak

Is this really an outbreak?

An outbreak is a series of disease events clustered in time and space. The disease events are usually new cases of a disease occurring at higher frequency than what is **normally** expected.

There are three ways to measure and describe disease frequency:

1. **Count the number of cases**

2. **Observe the pattern of cases**

3. **Calculate measures of disease frequency**

1. Count the number of cases

Sometimes knowing what is "normal" is a challenge in aquatic animals. For example, when the disease of interest occurs endemically, or we don't have much information about the expected level in the population of interest, confirming we have an outbreak can be problematic. It may come down to a judgement call. Other factors such as increased awareness and reporting of a disease may influence the number of new cases being notified.

Knowing what is normal is particularly difficult in wild populations. For example, the parasite *Bonamia exitiosa* which can cause mortalities on native oyster farms occurs endemically and without symptoms in wild native oysters in Victoria. The background level of infection in the wild through routine surveillance may be somewhere between 5 per cent and 20 per cent varying with season and other factors. If we have a report of 25 per cent infection in the wild population, is that an outbreak?

Photo. Beaufort Inlet (Photo courtesy of Marion Massam, DPIRD WA)

Photo. Native oyster farm in Port Phillip Bay (Photo courtesy of Tracey Bradley)

"Fish kills" are a specific type of outbreak occurring in the wild. Often, they are reported when there have been noticeable numbers of fish affected and as such can be considered an outbreak.

Emergency animal diseases represent a special situation where even one case requires particular attention until ruled out as a false positive or confirmed as a true positive. The same principles of outbreak investigation apply but we would not wait until we have a number of cases.

Hypothetical examples of emergency animal diseases:

- A known exotic disease agent enters a susceptible population e.g. Taura syndrome virus in Queensland (QLD) prawns

  OR

- A new or emerging disease agent e.g. a new variant of a current endemic disease *Yellow head virus 7* causing disease signs in QLD prawns

  OR

- An endemic disease agent enters a previously unexposed population e.g. *abalone viral ganglioneuritis* (AVG) in the South Australian (SA) wild abalone population (where the disease is not known to occur).

2. Observe the pattern of cases

How is the disease occurring in the population? There are three terms used to describe disease patterns and these terms are illustrated with "epidemic curves".

Endemic disease pattern

Cases occur regularly at a reasonably constant level e.g. *Amoebic gill disease* in Tasmanian salmon (Figure 2).
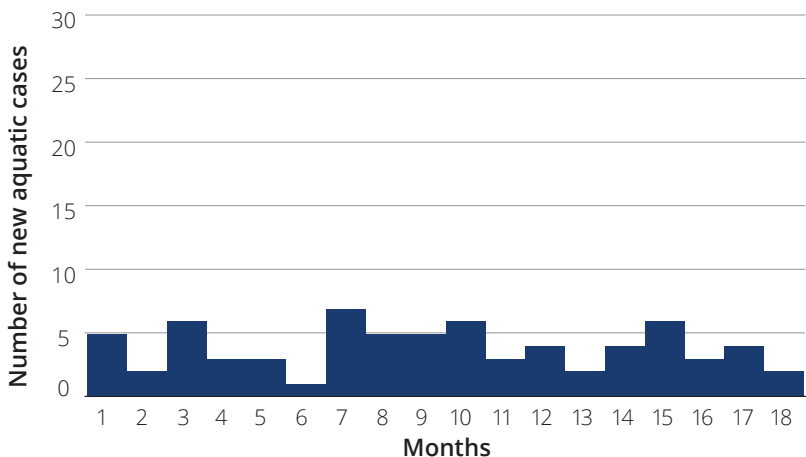
Figure 2. Epidemic curve of an endemic disease

Sporadic disease pattern

Cases occur infrequently and without any obvious pattern
e.g. *Epizootic ulcerative syndrome* (EUS) in Murray cod (Figure 3).
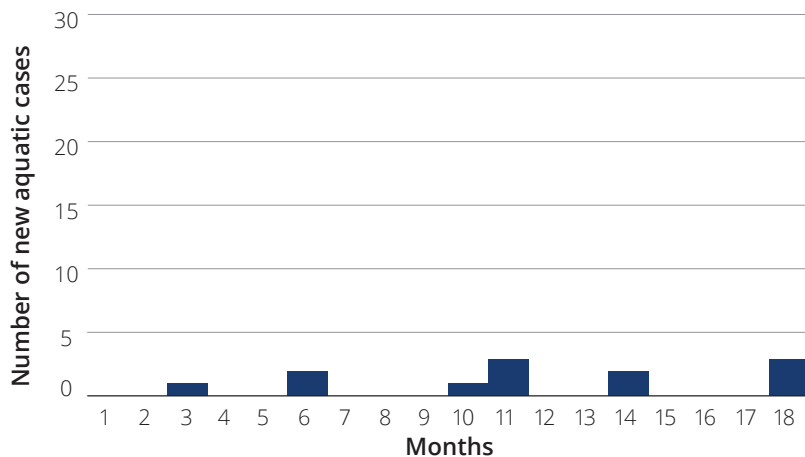
Figure 3. Epidemic curve of a sporadic disease

Outbreak/Epidemic[2]

Cases occurring in clear excess of what would be expected for the
population (Figure 4). If there were a number of farms infected with
the disease clustered in time and space we would more likely consider
this an epidemic.
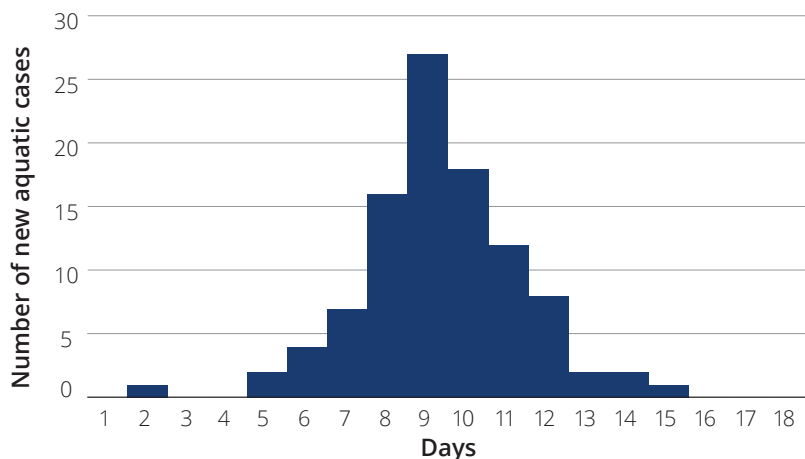
Figure 4. Epidemic curve of an outbreak/epidemic

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2 The term *outbreak* is often used interchangeably with *epidemic*.

3. Calculate measures of disease frequency

Now let's look at the **frequency** of disease compared with what would be expected in a similar group of animals under similar conditions.

There are two main measures of disease frequency and the difference between them relates to the time period involved.

The **prevalence** of disease is the proportion of individuals within the population at risk that have the disease at a **particular point in time**. This measure reflects the number of existing cases. Where we are using a mass screening diagnostic test, only one test is used to give us the prevalence of disease.

$$\text{Prevalence percentage} = \frac{\text{number of animals with a disease at a particular point in time}}{\text{number of animals in the population at risk at that point in time}} \times 100$$

Note. Animals that are vaccinated or previously exposed (and immune) are not included in the population at risk

Warning danger zone

Disease **incidence** is a more challenging area as we are measuring new cases of **disease over a period of time**. This is different to prevalence that is measuring existing cases at a single point in time. Incidence can be considered a dynamic measure whereas prevalence is a static measure. Incidence can help estimate the risk of disease in the future. For example, if we know that 1 in 10 batches of native oysters will be infected with bonamiasis (a parasite), when growing out a crop, the farmer can expect that there will be an approximate 10% risk of bonamiasis affecting crops in the future.

Cumulative incidence measures the proportion of the population at risk that develops the disease over a specified time period. Given it is a proportion it can be expressed as a percentage.

$$\text{Cumulative incidence} = \frac{\text{number of new cases of disease over a specified period}}{\text{number of animals in the source population at risk}} \times 100$$

The cumulative incidence does not account for situations where there is a large change in numbers of the population at risk (with additions and withdrawals). To handle such fluctuations, you can average the population over the time period (e.g. month) or reduce the specified time period as demonstrated in the "Happy Abs" example (see page 12).

We need to understand incidence because we often use a measure known as the **attack rate** when looking at disease outbreaks. The attack rate is a subtype of cumulative incidence and is the proportion of a specific population affected during an outbreak. Comparing attack rates in different groups can help us look at potential factors that may be responsible for causing the outbreak. This will be explored further in Step 7 (see "Step 7 Analyse the data" on page 46).

$$\text{Attack rate percentage} = \frac{\text{number of new cases since onset of outbreak}}{\text{total number of animals at risk at onset of outbreak}} \times 100$$

Photo. Southern rock lobster (Photo courtesy of Moon Cheese Studio)

1

CASE STUDY

Deaths in farmed abalone at "Happy Abs"

Abalone are dying on an onshore abalone farm "Happy Abs"[3]. The farm has 1000 tanks, and predominantly grows out stock but also has broodstock and a hatchery. The initial signs are sick and dying abalone and the cause was believed to be poor water quality. Only two tanks of broodstock are initially affected with increased mortality.

The expected level of mortality in the broodstock is very low, usually less than **1 per cent per month** (this is an approximation of monthly cumulative incidence). We have two tanks of broodstock with 156 in one tank and 97 in the other on 11 June. Table 1 records the number of dead abalone found every day in these two tanks. Note the change in population of the tank as the dead abalone are removed from the "at risk" group (total abalone). Most of the other tanks on the farm recorded zero mortalities during this period.

This is only five days of **daily** cumulative incidence data for two tanks but comparing this with the average **monthly** cumulative incidence data of less than 1 per cent, we can see that the deaths in the each of the two tanks is clearly well beyond the **expected** normal disease incidence for the farm.

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3   This is a fictitious example.

	Tank M32				Tank M33		
Date	Mortalities	Total abalone at risk	Daily incidence %	Mortalities	Total abalone at risk	Daily incidence %
11/6	0	156	0	0	97	0
12/6	3	156	1.9	0	97	0
13/6	9	153	5.9	0	97	0
14/6	18	144	12.5	5	97	5.2
15/6	27	126	21.4	12	92	13.0
16/6	32	99	32.3	11	80	13.8
Totals	89			28		

Table 1. Mortality data for two tanks at "Happy Abs"

We can state with certainty that **this is an outbreak**. At this stage we have not developed a case definition, but we can say the number of *dead* abalone is well beyond what is expected.

The **attack rate** for dead abalone in these two tanks between 11/6 and 16/6 is:
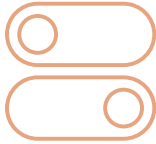
Tank M32	Tank M33
$\dfrac{89}{156}$ **= 57.1 per cent**	$\dfrac{28}{97}$ **= 28.9 per cent**

Attack rates are useful when we are looking at exposure factors and will be further explored in Step 7 (see "Step 7 Analyse the data" on page 46).

STEP 2

**Define
a Case**

What is a case?

Defining a case is an essential step in any outbreak investigation.

> **Case =** the unit of interest[4] that meets a set of standard criteria for having the disease or outcome of interest.

The case definition can be broad or more specific depending on what is known about the disease under investigation.

Most importantly the case definition should differentiate the animals affected by the disease under investigation from unaffected animals and those that may be suffering from other conditions (particularly endemic diseases) that occur in the population of interest.

The case definition may include the species and age of affected animals, presenting signs, response to treatment or other characteristics. If a laboratory diagnosis is available this should be included in the case definition with as much detail as possible (including the type of test undertaken).

Remember the aim is to find the source of the problem whether we have a diagnosis or not.

As more is known about the characteristics of cases, the definition can be tightened and made more specific.

Perfecting the case definition is an art. It is important to balance a broader case definition, which may include animals that are not affected by the disease of interest but may have a similar condition, with becoming more specific and risk not including legitimate cases. The case definition may apply at an individual animal, tank, farm or catchment/water body level.

Photo. Spiny sea urchins (Photo courtesy of Mooncheese Studio)

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4 Refer to page 43 for the definition.

CASE STUDY

Returning to our farm "Happy Abs"

You have now been called out to the farm to investigate as the situation is not resolving and deaths continue to occur. You collect a range of samples (affected and unaffected animals) to take to the laboratory (see "Sample submission" on page 67).

Looking at the data, we have confirmed there is an outbreak on the farm based on the much higher than usual incidence of mortality and comparative attack rates in two tanks. We were initially working with the very broad case definition of "dead abalone". When you arrive on the farm you note the following clinical signs in affected abalone; enlarged mouth, a curled foot and protruding radula (tongue) see Figures 5 and 6.

The new **animal level** case definition is updated to – **a deceased abalone displaying a curled foot, enlarged mouthparts and protruding radula**. This definition is appropriate if all abalone are displaying these clinical signs. A broader definition could include all dead abalone and live abalone with the clinical signs if we know that some abalone will die very quickly without signs. At a tank level, a "case tank" could be defined as "a tank having daily mortality over 2 per cent for two or more consecutive days". The specific clinical syndrome could also be included in the tank level definition.

If, and when we have a laboratory confirmed diagnosis, the new case definition could be updated to include details about the laboratory diagnosis including the type of test conducted (pathology, molecular test etc). If we wish to include sub-clinical animals that are testing positive to a disease agent, the clinical symptoms may not be a necessary part of the case definition. For example, in the circumstance where a virus (virus X) is a known cause of disease, we could define a case tank as having "one confirmed positive of virus X by PCR test".

Figure 5. Healthy abalone from "Happy Abs"

Figure 6. Unhealthy abalone from "Happy Abs"
Photos courtesy of Agriculture Victoria

STEP 3
Collect data

What can we find out about the cases?

Accurate collection and recording of data is a critical step in an outbreak investigation. It can be the most challenging, yet rewarding, part of the process.

Farm data

How farmers collect and record data varies considerably. A Murray cod farm stocking a large dam that stores water for crops may record very limited data. It may be difficult for the farmer to estimate the number of mortalities in a pond where there are high rates of cannibalism when fish die.

Large, capital – intensive corporate farms such as those in the Tasmanian salmon industry, or abalone enterprises with farms across several states will have large volumes of data measuring a range of variables including mortality rates and growth rates.

Data will be available from farm records, interviews, farm observations and assessment of stock and their environment on your farm visit. Additional data will come from any laboratory investigation.

SOME TIPS ON COLLECTION OF DATA

- Collect information on cases **and non-cases**.
- Wherever possible access objective measures of data rather than rely on farmer interpretation e.g. water quality data, spreadsheets of recorded mortalities.
- Record the date of the first case with compatible clinical signs.
- Use consistent identification across farm records and laboratory submission forms.
- Avoid manually transcribing data to reduce errors.
- Be forensic and thorough but with an open mind – it is up to you to objectively collect, synthesise and assess all the data.

Photo. Abalone (Photo courtesy of Nick Savva, AAGA)

Wild population data

Collecting data to aid in the analysis of disease in wild populations is much more challenging than reviewing records on a farm.

Depending on the fishery, the level of knowledge about existing stocks will vary considerably. A commercially valuable and regulated species may be carefully managed by authorities or the local industry body with robust estimates of existing biomass. This data may have been captured by regular and proven quantification methods such as transect counts used in the abalone commercial catch industry.

For highly pelagic species estimating fish populations is much more difficult. The level of mortality in the pilchard die-off of 1995 will never be accurately known.

Calculating the denominator on farms and in the wild

Working out the number of fish in the population at risk can be challenging! Farmers will often know the approximate number of fish in a culture unit. Where this number is quite uncertain, an approximation will need to be made and treated with caution. We need to assess the accuracy of calculating the biomass and therefore number of animals in a unit and consider how often counts are undertaken and potential sources of bias.

In wild populations, best estimates from industry/government sources, transect counts, or researchers, can be used as outlined above.
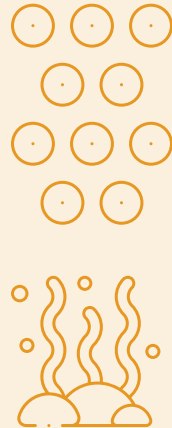
Tracing movements in a disease outbreak

(This is usually more relevant to farm outbreaks)

On the farm

The initial farm visit should include collection of data of recent movements (traces) onto or off the farm. Movements to consider will depend on the disease organism (if known) and how it is spread. Potential movements to investigate include aquatic animals (all stages of life, live and dead), water, vehicles, wildlife, feed, humans and equipment (including boats).

Indirect transmission or spread

- Water droplets
- Dust >5 microns

- Birds, aquatic mammals
- Other aquatic species e.g. sea lice

- Vehicles e.g. boats or trucks
- Personnel e.g. staff and visitors
- Equipment e.g. brush, net

Mechanical or biological vector

Airborne

Aquatic animal

Vehicle (Fomites)

Vertical

Exposure via water

- Spawning

- Direct contact with other infected animals
- Contact with vomit/faeces
- Cannibalism
- Contact with infected processing waste
- Incoming infected water

Direct transmission or spread

Figure 7. Potential sources for infectious diseases in an aquatic setting (adapted from Oidtmann et al, 2013) see "References" on page 78.

Backward (source) tracing will provide information regarding the potential introduction of disease agents onto the farm. All movements onto a farm do not represent the same risk of disease transfer. **Live animals are generally considered the highest risk for transfer of infectious diseases and should be prioritised in the tracing process**. Figure 7 illustrates a range of sources of infection both indirect and direct.

Forward (spread) tracing focuses on the potential spread of disease to other parts of the farm, other farms and the wild (where the disease is not already present in the wild).

In the wild

Undertaking tracing in the wild is much more difficult than on a farm. Many different stakeholders may need to be contacted – researchers, government, industry bodies, etc. The source of an infectious agent in a wild aquatic animal population may include movement of stock into a fishery for rebuilding purposes, live/dead bait use and infected ballast water from cargo ships. Forward spread is often unpredictable and will be influenced by factors such as ocean currents, supply chain arrangements, movements of infected animals and equipment.

When did the disease enter the population?

If the **incubation period** is known for a disease agent, a **tracing window** can be constructed and movements during this time intensively investigated.

Incubation period is the period of time from exposure to the infectious agent through to when clinical signs are seen.

A tracing window refers to the most likely period of time during which the disease could have been introduced to an area (tracing window for source), or the most likely period of time during which the disease may have spread to another area (tracing window for spread). Figure 8 illustrates the tracing window for source and spread relative to the incubation period of a disease.
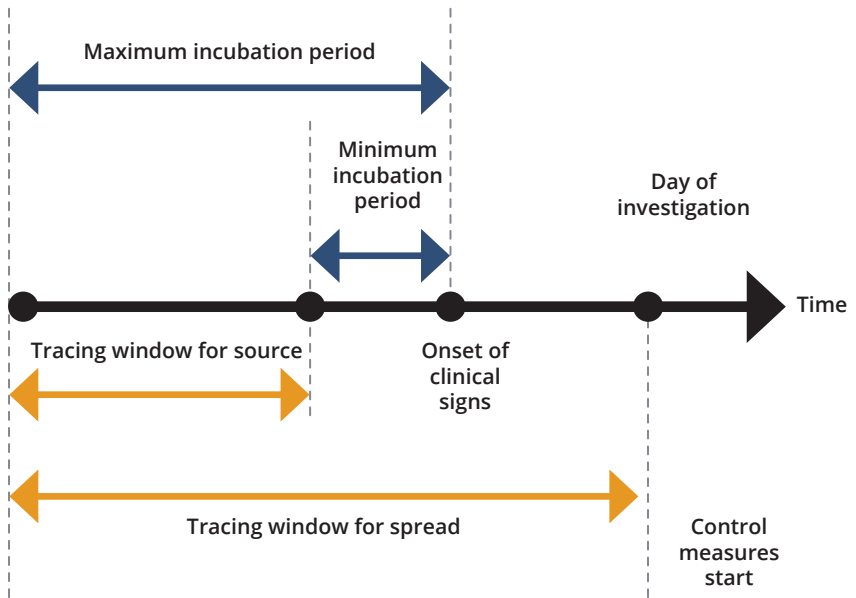
Figure 8. Tracing window for an infectious disease source and spread

Surveillance

Following review of our outbreak data on a farm or in the wild, surveillance of other local farms, catchments, marine areas or the rest of the state may be appropriate. Further resources on surveillance are provided towards the end of the guide.

Surveillance is a systematic series of investigations of a given population of aquatic animals to detect the occurrence of disease which may involve testing samples of a population.

CASE STUDY

Returning to our farm "Happy Abs"

Back on the farm, now you have collected some diagnostic samples, it is time to look at the farm data available. This includes the farm map, recent spreadsheets with details of individual tank mortalities and production, water quality data, records of stock movements onto and off the farm, information about the on-farm processing plant, any treatments provided etc.

You turn your attention to tracing movements onto and off the farm (source and spread). A long list is compiled of all the possible sources of infection for the clinical syndrome seen.

The first clinical signs were seen in M32 broodstock on 12/6. You discover that 10 broodstock were brought onto the farm from an interstate farm "All About Abs" which is a farm connected through the parent company. These abalone arrived on 9/6 and were placed into tank M32. You also note that cleaning equipment is shared between tanks M32 and M33 (and others). The farm pumps water at a rate of 1100 litres per second but there is no known disease in the wild within 500 kilometres of this farm.

Given the infection appeared to originate in the tank of the recently introduced broodstock, a high priority for investigation will be the traceback enquiries to the interstate farm "All About Abs".

Photo. West Coast Oysters Coffin Bay (Photo courtesy of Oysters SA)

STEP 4

Describe the temporal pattern of new cases

When is the outbreak occurring?

The occurrence of new cases of the disease over time is described as the temporal pattern of disease.

Time based information is collected and organised to find answers to the following questions:

- **When did the outbreak begin?**

- **What is the pattern of disease over time?**

- **What is the most likely period of introduction/ exposure? (see Step 3)**

- **Are there seasonal or cyclical patterns of disease apparent over longer periods of time?**

The epidemic curve

Epidemic curves are very important in the initial stages of an outbreak and can be created using spreadsheet software such as "Microsoft Excel" or specialised programs. The curve provides a visual representation of the magnitude of the event and the rate at which new cases are occurring. They also help monitor efficacy of control measures.

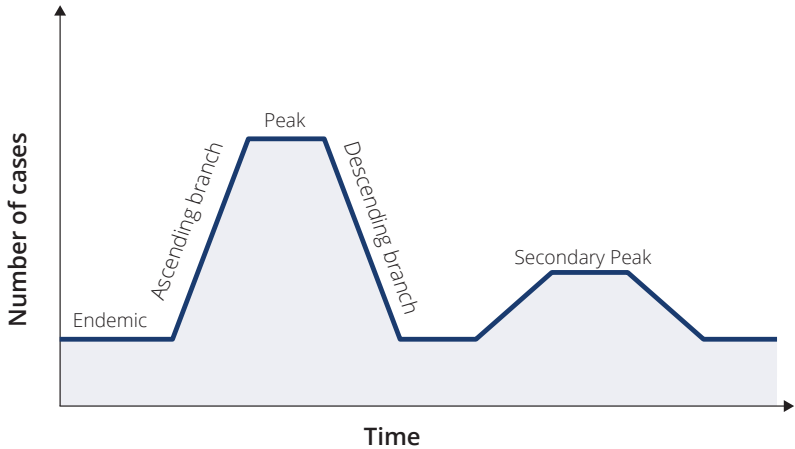There can be five stages in an epidemic curve as shown in Figure 9.

Figure 9. Stages of an epidemic curve

What influences the appearance of the epidemic curve?

Rapid rise in the ascending branch:

- Where the transmission is fast and effective, for example a highly infectious disease with a short incubation period coming onto the farm in the water.
- Exposure of a group of animals to a toxin, for example a contaminant affecting a number of fish in the wild within a short period of time (see *point source epidemic* on following page).

Gradual rise in the ascending branch:

- A disease with a long incubation period
- Low level of infectiousness of the disease, for example – a virus that requires direct contact in a dispersed, sessile (attached) animal.
- Disease requires an intermediate host, for example whirling disease (*Myxobolus cerebralis*) requires the mud worm to complete the life cycle.

The length of the plateau and descending branch will be influenced by factors such as the availability of susceptible animals, the time over which susceptible animals are exposed to the infection source and the minimum and maximum incubation periods of disease. Immunity across the group also affects the shape of the curve and will be influenced by previous exposure to the disease agent or interventions such as vaccination programs.

For outbreaks extending over longer periods, a seven-day rolling average of the epidemic curve can be useful.

Secondary peaks are seen when there are new, susceptible introductions into the population at risk, movement of infected animals into a new area with susceptible animals or a change in mode of transmission.

HINT

When producing the curve, try several different time intervals to best view the pattern and avoid missing secondary peaks.

A guide is to make the time interval ⅛ to ¼ of the estimated incubation period. E.g. EUS has an incubation period of 10 days, the number of new cases should be measured every two days.

Outbreaks can be referred to as being either:

- **"Common" or "point" source:** where a large number of cases result from exposure to a common source agent within a short time period. This pattern is generally associated with food or waterborne agents such as toxins or when a large proportion of the population is exposed to an infectious disease simultaneously.
- **"Propagating" epidemic:** where the disease agent spreads either directly or indirectly from an infected host to susceptible animals.

Common source
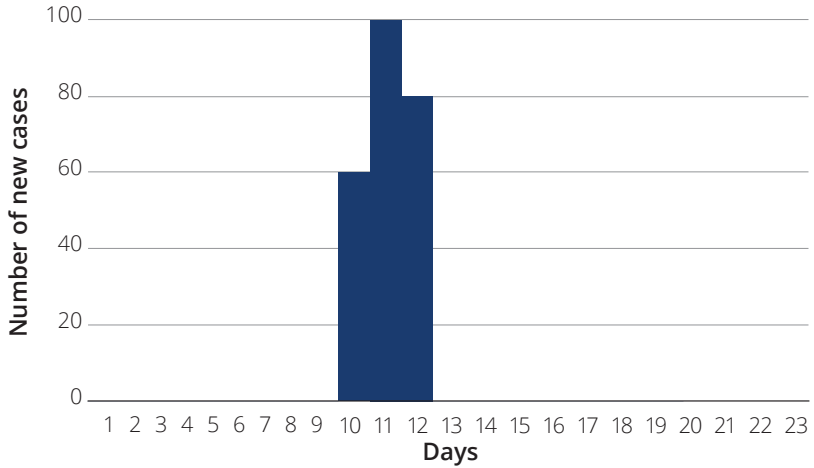
Propagating epidemic
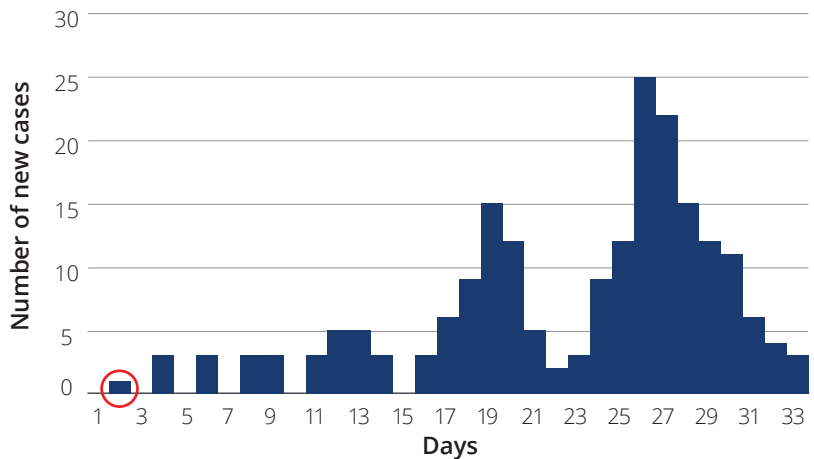
Figure 10. Temporal distribution of common source vs propagating epidemic

A propagating epidemic curve may appear similar to a common source epidemic curve if the infectious disease has a very short incubation period. Likewise, a common source epidemic curve could appear as propagating with repeated introduction of a toxin over a prolonged period of time.

The index case is circled in red in the propagating epidemic curve (Figure 10). Finding this case is important in identifying the source. In some outbreaks the index case may be an individual case and the time period to the main peak will be the incubation period.

The **index case** is the first diagnosed case of an outbreak.

At an individual level, disease time periods are described as below in Figure 11 but can vary by agent and individual.

Shedding of agent (this may occur prior to onset of clinical signs)

Infectious period

Latent period

High risk period

Time

Incubation period

Exposure to disease agent

Onset of clinical signs

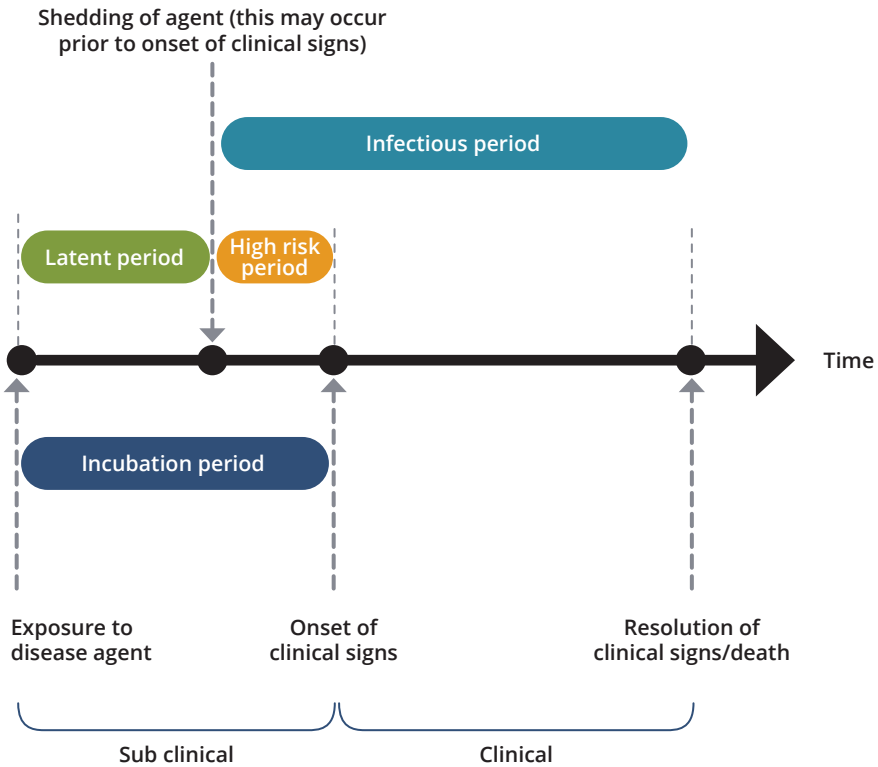Resolution of clinical signs/death

Sub clinical

Clinical

Figure 11. Theoretical timeline of elements of a disease in an aquatic animal

Disease time periods

Latent period

For infectious diseases it is the time period from exposure to when the infectious agent is shed.

Infectious period

The time period during which an individual can spread disease to another animal. An animal may be clinically affected but not infectious, or, infectious but not clinically affected.

High risk period

The time period during which the animal is shedding infectious disease agent but there are no clinical signs of disease.

Incubation period

The time period from exposure to infection through to when clinical signs are manifested. This may be referred to as the **induction period** in the event of a toxic insult such as chemical contamination.

Subclinical

When an animal is infected but there are no overt clinical signs.

Clinical

When there are overt external signs or behavioural changes due to infection.

4

CASE STUDY

Let's go back to our farm "Happy Abs"

We are now 20 days into the event and have a laboratory confirmed diagnosis of s*leepy abalone disease* (SAD – a fictitious disease) caused by *sleepy abalone disease virus* (SADV).

We can now refine our case definition "*A deceased abalone displaying a curled foot, enlarged mouthparts and protruding radula for which the SAD virus has been detected by qPCR*" (a specific molecular test).

Usually, we would have positive qPCR results prior to clinical signs as the test is very sensitive. If we wanted to increase the chance of including subclinical animals in our diagnosis, we could incorporate any positive laboratory result (even without clinical signs) into our case definition.

We collect data from all the tanks (Table 2) and construct an epidemic curve (Figure 12). We know from previous outbreaks that this disease is water borne, highly virulent and difficult to kill using standard disinfectants. The disease has a short incubation period of around three days. We had already instituted quarantine of infected tanks based on the rapid spread of the disease. Once we had the diagnosis confirmed by the laboratory, we commenced an emergency harvest of infected tanks which will take some time to complete.

From the epidemic curve we can see that the index case was on 12 June, the peak of the outbreak was 19 June with 14 cases. The shape of this curve is suggestive of a propagating epidemic and the duration of the outbreak was 19 days. The long tail of the curve may imply that there was a lot of movement of abalone prior to quarantine being instituted or the control measures were slow to take effect.

Date	Tanks with >2% mortality		Date	Tanks with >2% mortality		Date	Tanks with >2% mortality
10/06	0		17/06	8		24/06	9
11/06	0		18/06	10		25/06	8
12/06	1		19/06	14		26/06	7
13/06	2		20/06	12		27/06	3
14/06	3		21/06	11		28/06	3
15/06	3		22/06	11		29/06	2
16/06	5		23/06	10		30/06	1
						Total	**123**

Table 2. Tank level mortality data for "Happy Abs"
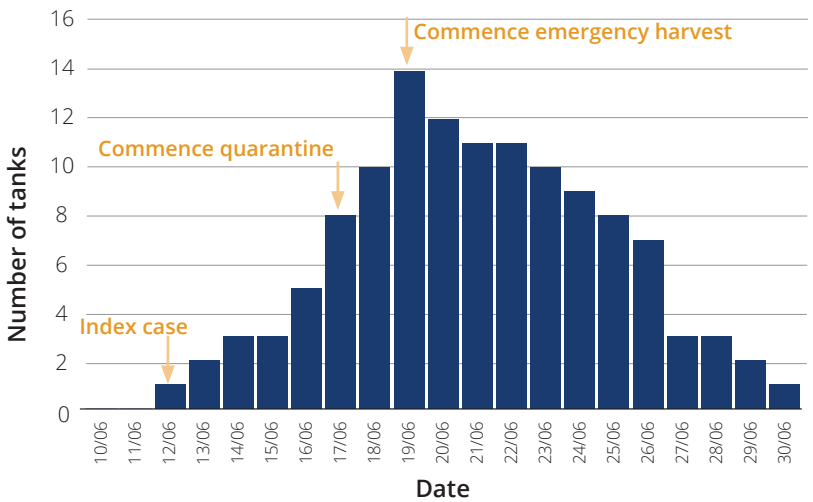
Tanks with greater than 2% mortality

Figure 12. Epidemic curve for diseased abalone at "Happy Abs" abalone farm and associated data

Information on how to create an epidemic curve is provided in the "Resources" appendices on page 76.

Predicting the spread of disease

The temporal information displayed in the epidemic curve is useful for analysing the outbreak as it is happening.

However often we want to know what the situation will be tomorrow or next week for planning purposes. The Estimated Dissemination Ratio (EDR) is useful in assisting with this planning. In cattle outbreaks of FMD, the EDR at a farm level has been used to predict the trajectory of outbreaks and assist with planning control approaches. The EDR is calculated by the number of new cases in a defined time period (e.g. seven days) divided by the number of cases in the previous defined period.

$$\text{EDR} = \frac{\text{cases in x days}}{\text{cases in previous x days}}$$

Calculating the EDR (at a tank level) for "Happy Abs" is relatively simple. Let's assume it is day 11 of the outbreak and all the data is in for the previous 10 days. We are interested in what has happened in 5-day time periods.

The EDR will be 55/14 = 3.9. This is calculated by summing the case tanks from day 6 – 10 of the outbreak divided by the case tanks from day 1 – 5. An EDR >1 indicates the epidemic is continuing to grow and an EDR <1 indicates the epidemic is declining.

If we calculate the EDR for days 11 to 15 and divide by the previous 5 days we calculate the EDR as 45/55 = 0.8. This figure indicates the outbreak is declining. Recall that given this event is only occurring on one farm it is an outbreak, not an epidemic.

Care is required in outbreaks with few cases as very small changes in case numbers will result in a large apparent change in the EDR. Five days was chosen in this instance, but a longer period (such as a week) could have been selected.

Photo. Tasmanian salmon farms. (Photo courtesy of Huon Aquaculture)

4

STEP 5

Describe the spatial pattern of new cases

Where is the outbreak occurring?

The spatial pattern of an outbreak describes the distribution of disease by place.
An outbreak usually refers to cases not only clustered over time but in an area.

The scale of the spatial distribution can vary widely. Disease may be occurring on a single farm within tanks or ponds, across a number of farms, in rivers in a catchment or across countries with contiguous waterways or oceans.

What can the spatial pattern tell us?

Spatial patterns demonstrated on a map can provide very useful information on exposure and transmission mechanisms in an outbreak. Even simple maps can indicate spread within a farm due to water movements/staff activities/sharing of equipment etc. Infected tanks or ponds located close to the water inlet may indicate spread through incoming water. Tanks infected at a distance from the inlet may indicate the disease is associated with water quality factors such as lower oxygen levels.

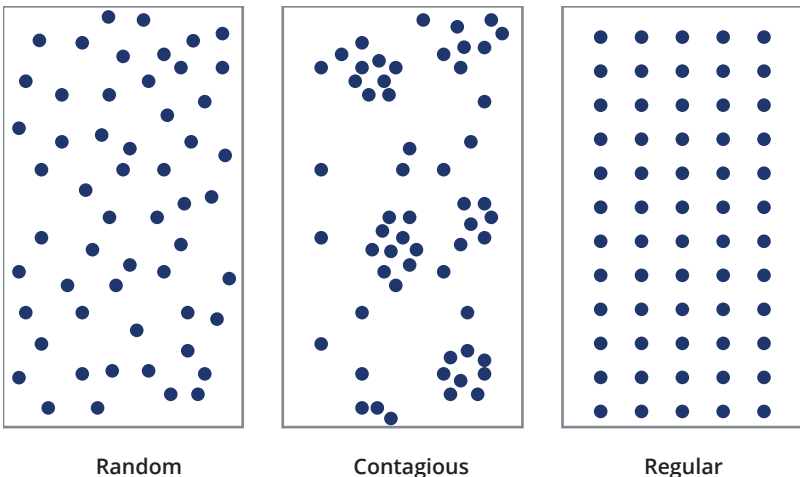Some examples of the types of spatial patterns are illustrated in Figure 13.

| Random | Contagious | Regular |

Figure 13. Examples of different types of spatial pattern (Southwood, 1978)

Photo. Smoky Bay Pacific oyster coast leases (Photo courtesy of Oysters SA)

How to map the outbreak varies with the situation and may include:

- A simple hand drawn map of a farm.
- An overlay on pre-existing software such as "Google Earth".
- Manipulation of existing farm mapping data – this may include paper-based maps.
- Setting up a diagrammatic map through a program such as Microsoft Excel (see Figure 14).
- Collecting GPS coordinates and mapping out the outbreak through free or proprietary mapping software products.

The scale of the map needs to be appropriate to the disease. When looking at several farms across a state or country, make sure both infected and uninfected farms are displayed.
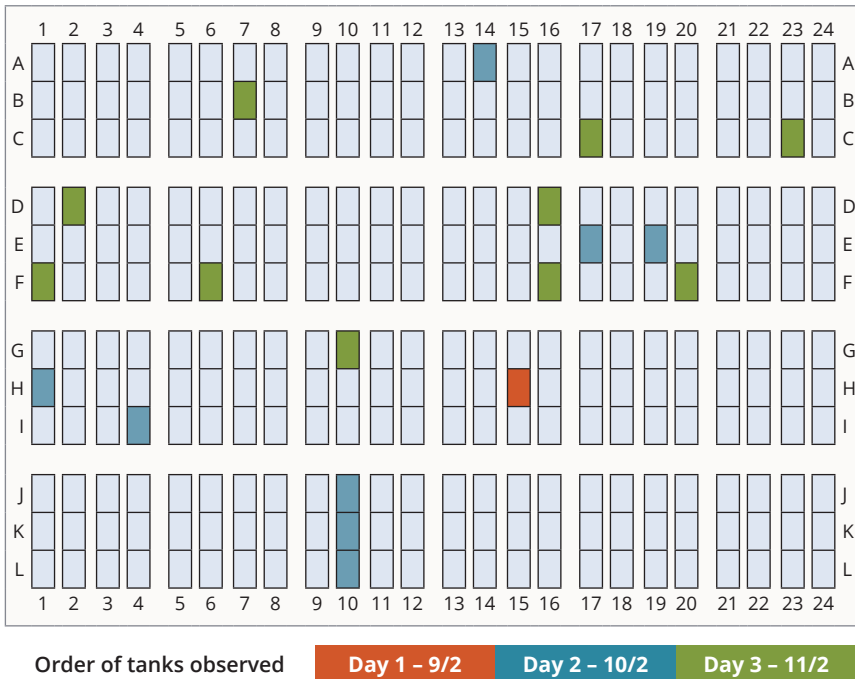

Figure 14. Progress of mortalities through a fish farm. There is no clear pattern discernible. This is a good example of an outbreak with a **random** pattern and was due to a water quality issue
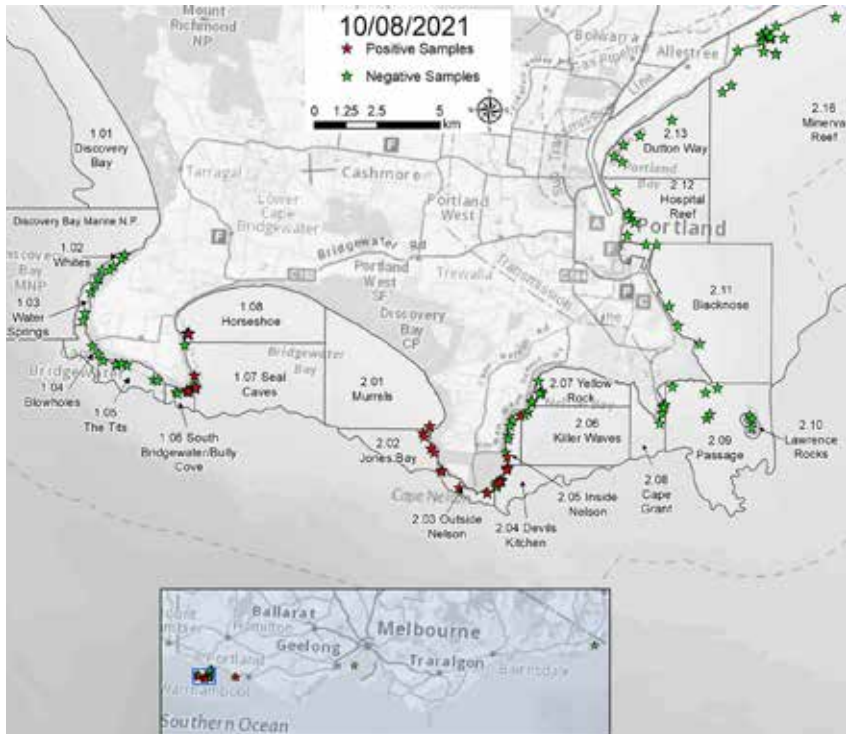
Figure 15. Distribution of HaHV-1 virus in wild abalone in the western zone of Victoria. This map illustrates clustering of positive cases in a **contagious** spatial pattern. Map courtesy of Agriculture Victoria

Without mapping all farms, you run the risk of assuming clustering in one area is due to a particular risk factor in the area when it may be that the cluster occurs where all the farms are located. Figure 14 demonstrates a random pattern of disease on a farm and Figure 15 demonstrates clustering of a contagious disease in wild abalone on the Victorian coastline. A regular spatial pattern may occur when a treatment or intervention is applied in a systematic manner, for example every second tank in the nursery is fed a contaminated food inadvertently.

Knowing how far a disease can spread within a specified timeframe is crucial for implementing successful control measures.

CASE STUDY

## Let's go back to our farm "Happy Abs"

Let's return to our case study at "Happy Abs" farm and *go back in time* to see what happened with the spatial pattern of the outbreak.

Initially we found M32 with newly arrived broodstock was the likely index tank. M33 – M35 tanks were believed to be infected via shared cleaning equipment given their proximity.

Staff then noted that there were also abalone showing clinical signs in tanks in the grow out part of the farm (S42 – 45). It is apparent from the farm map that the disease has spread to a completely different part of the farm.

On questioning, we discover that a junior staff member moved stock from M34 to the S42 – S45 tanks as part of the farm scheduled grading process. Unfortunately, the disease was then moved through tanks with cleaning equipment prior to quarantine being instituted.

Mapping out the infected tanks (Figure 16), particularly as they become infected can help us understand how disease is moving around the farm.
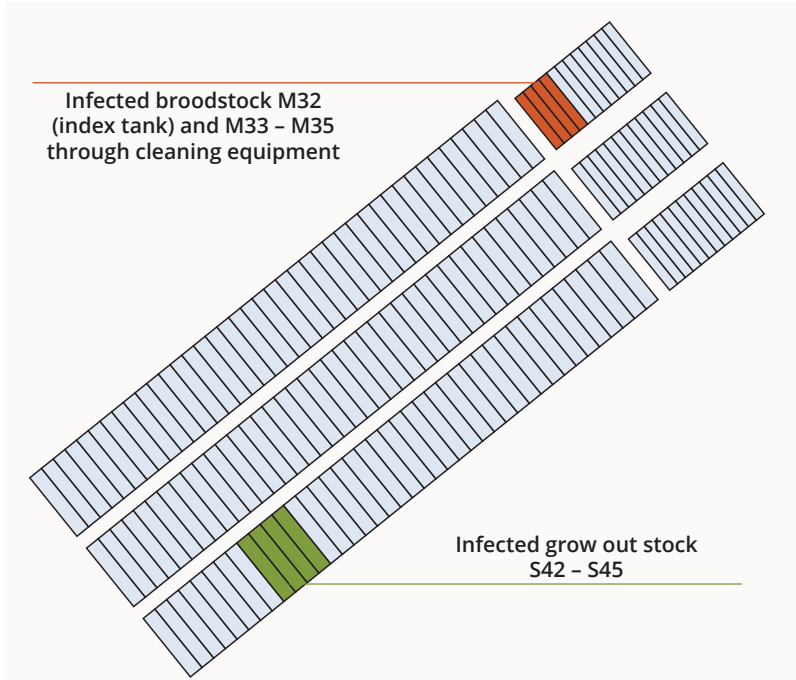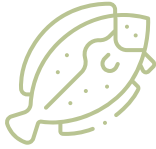
Figure 16. Map of infected tanks at "Happy Abs" early in the outbreak. This is a partial representation of the farm.

STEP 6

Describe the population pattern of new cases

Who is this outbreak affecting?

Examining patterns of disease in populations of animals can help us understand the cause of disease and how to control it. We need to establish the unit of interest that we are concerned with in an outbreak. Recall in Step 2 we had already defined a "case".

In our example of "Happy Abs" we have defined the unit of interest as the tank. This is because the farm is logically arranged and managed at a tank level.

Unit of interest =	the biological unit of primary concern in an epidemiological investigation. This may be the animal, pond/tank or the whole farm in aquaculture.

Characterising an outbreak by affected groups of animals will help to determine which group of animals are most at risk of disease.

The animal specific factors in Figure 17 (next page) may relate to susceptibility to disease or opportunities for exposure to disease.

Photo. Koi carp
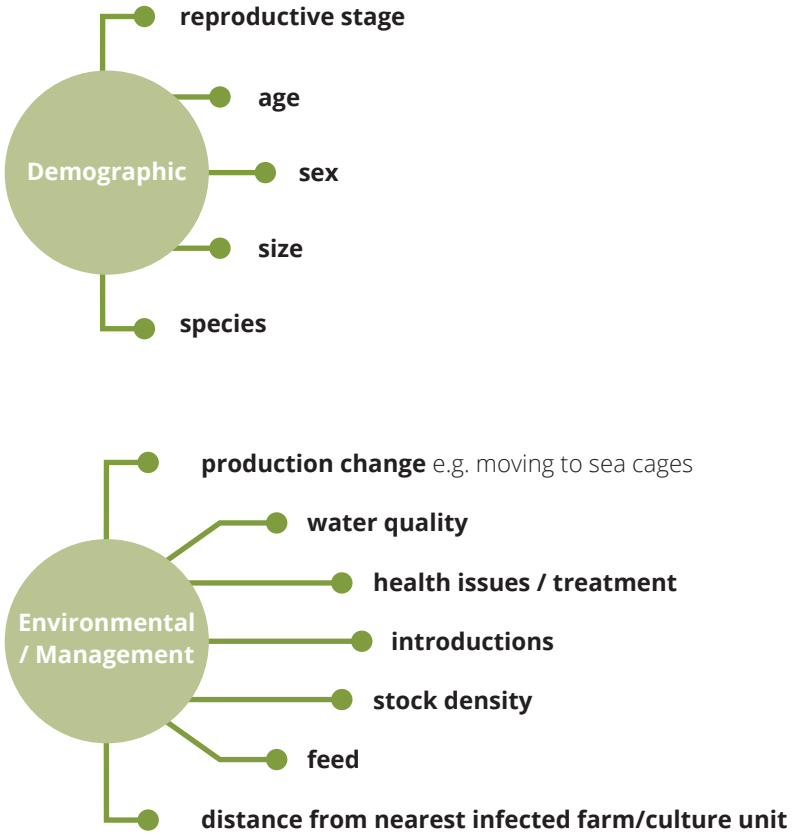
Figure 17. Demographic and other factors potentially associated with a disease

If data is available on the individual, analysis of age or sex is usually evaluated first.

This information is ideally collected at the start of an outbreak and will be used to create **factor specific attack rates** (see Step 7 "Analyse the data" on page 46).

CASE STUDY

Returning to our farm "Happy Abs"

Looking at our farm "Happy Abs", we can investigate the demographic factors species, reproductive stage, age and size (the last two are often correlated).

For the potential environmental/management risk factors associated with SAD, we have established from farm data that the water quality and feed are consistent across the farm, there have been no changes in management nor health problems reported either within the farm or outside the farm.
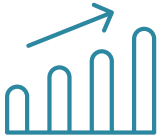
We are aware that there has been a recent introduction of broodstock as discussed earlier, but aside from this, there are no other movements onto the farm.

The farm does have some variability in stocking rate which will be further investigated in Step 7.

6

STEP 7

Analyse the data

What is the data telling us?

The epidemiological approach to outbreak investigations works on the principle that cases of disease do not appear randomly but occur in patterns in the at-risk population.

Whether an animal becomes diseased or not is influenced by a range of possible factors relating to the animal (such as age or species), the agent (strain virulence and mode of transmission) and the environment.

Measures of association with a potential risk factor

We used disease incidence and attack rate in the initial stages of our outbreak investigation on "Happy Abs" to determine if an outbreak was occurring. Recall from Step 1 that the attack rate is a subtype of cumulative incidence.

We will now use attack rates to compare and evaluate specific factors increasing the risk of disease. These may include the demographic and management factors outlined in Step 6. This evaluation is usually conducted by setting up an attack rate table. An example of a single factor attack rate table is given in Table 3 where we have an unknown disease and have calculated the attack rates as a percentage for each level of the factor (being age). Forty of 100 juveniles have the disease and thus an attack rate of 40%. This is higher than the attack rate for adults and so, we can conclude that juveniles have a higher risk of disease.

Factor	Diseased	Total	Attack rate
Juveniles	40	100	40%
Adults	5	100	5%

Table 3. Single factor attack rate table

Another important measure of association between disease and a factor is the **relative risk**.

> **Relative risk** is an estimate of how much more likely disease is to occur in the group exposed group to the factor of interest compared to the unexposed group

The relative risk is calculated by dividing the attack rate in the exposed group by the attack rate in the unexposed group. The unexposed group is by convention, called the reference group and has the lowest attack rate. In the example given, the relative risk would be 8 (40/5). This can be interpreted as "juvenile animals were at eight times the risk of having disease than adult animals".

The higher the difference in attack rates and therefore the relative risk, the more important the specific risk factor is in increasing the risk of disease.

- When relative risk = 1   there is no association between the risk and disease
- When relative risk > 1   there is an increased association between risk and disease
- When relative risk < 1   the risk factor is potentially protective

It is important to recognise that these are measures of the biological importance of a potential risk. Statistical significance tests and confidence intervals will identify whether the observed result is likely to be due to chance or not. Further analytical methods are beyond the scope of this guide.

Traces – what do they tell us?

In our example at "Happy Abs" the tracing of a broodstock movement from an interstate farm shortly before the outbreak began was identified as a high-risk activity. If disease is detected at the originating farm ("All About Abs") then this would be compelling evidence on the source of infection.

The recent rise in prominence of molecular epidemiology has been a useful tool assisting with tracking the source of disease. Genotyping viruses and other pathogens has revolutionised this area. If we wanted further evidence on the origin of the "Happy Abs" virus, genotype "matching" with virus isolated from "All About Abs" could provide this.

In more complicated situations, network diagrams can be constructed to visualise the connections between tanks/farms/sites. In the below example (Figure 18) adapted from an outbreak of infectious salmon anaemia (ISA) in the Shetland Islands, the direction of arrows indicates the movement of fish, equipment, personnel or vehicles.
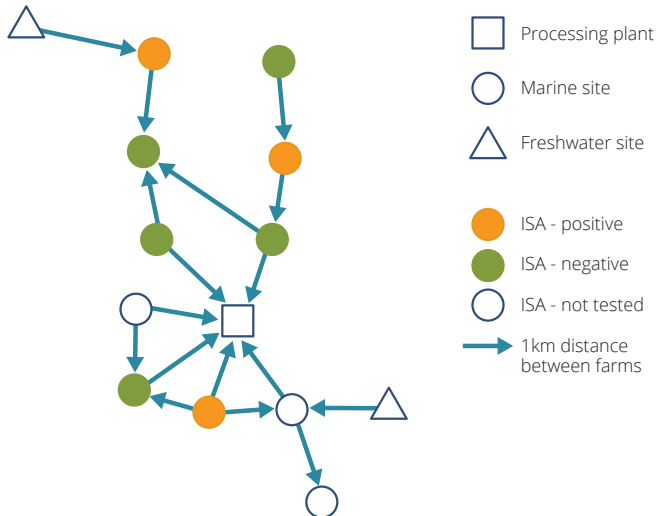

Figure 18. A network diagram for an outbreak of ISA in the Shetland Islands (adapted from Murray et al, 2010). Distances between sites is hypothetical

The arrows may be uni or bidirectional depending on the activity. The diagram indicates that all infected sites were disconnected from one another through movement of stock/equipment etc when the traces were reviewed. Site to site spread via ISA movement in seawater was suspected in this example.

The distance between the sites modified for this example is one kilometre; which is a plausible distance for water borne movement of the disease agent between farms.

Network diagrams combined with hydrographical modelling can assist in predicting the spread of a disease agent in the marine environment.

Once a network diagram has been constructed movement controls can be placed at strategic points to minimise the spread of disease.

Prioritising traces

Not all traces carry the same risk of disseminating infection (in the case of an infectious pathogen). Figure 19 demonstrates different prioritisation applied to different pathways. Mathematical probabilities can be applied to these risks but are beyond the scope of these guidelines.
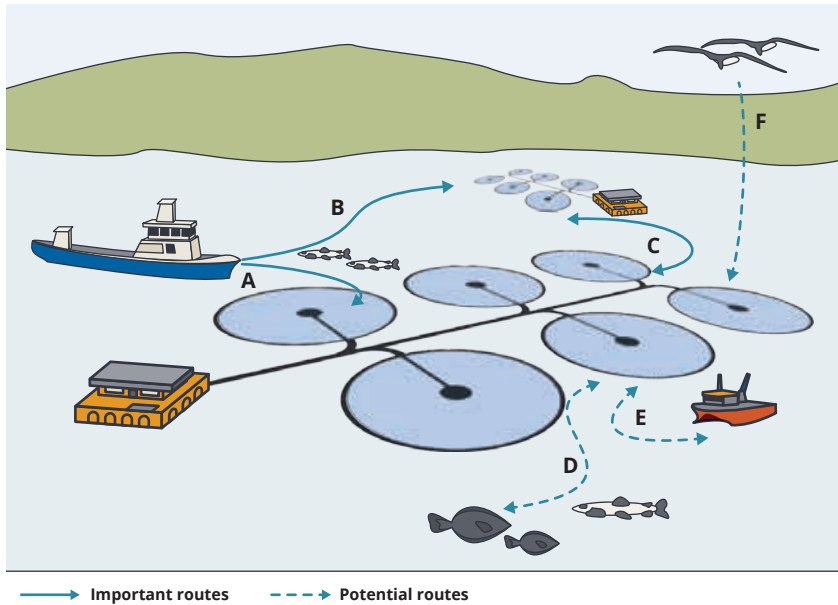
Figure 19. Diagrammatic representation of routes of infection for the introduction of infection onto salmon farms (Source Pettersen et al, 2015)

Routes for the introduction of infectious diseases into salmon farms

A: Sea transfer of infected smolts – high priority

B: Well boat traffic carrying infected fish – high priority

C: Water currents transporting disease agents – hydrodynamics can be considered for disease transmission

D: Wild fish, sea lice and escapees acting as disease vectors- cannot be traced but need to be considered for disease transmission

E: Traffic of farm workers, equipment and service boats for sea cage maintenance – low priority

F: Sea birds acting as disease vectors- cannot be traced but need to be considered for disease transmission.

CASE STUDY

Returning to our farm "Happy Abs"

We have now collated a large volume of data for "Happy Abs" and **the outbreak is over**. We know that there are 1000 tanks on the farm and 123 tanks in total were affected. The unit of interest is the tank given that all animals within a tank can be considered to be managed as a single group.

We discussed attack rates in Step 1. We have calculated factor – specific attack rates for two identified factors and have created the attack rate table (Table 4). When there are multiple categories for a risk factor then one group of animals must be chosen as the reference and others compared to this in a pairwise fashion (i.e. A vs C and B vs C, then A vs B only if required).

The reference group may be chosen based on a biological justification that makes sense, or due to it having the lowest attack rate. Traditionally, comparison is made with the lowest attack rate.

Tank level factor	Levels	Diseased	Total tanks	Attack rate	Relative risk
Species	Greenlip	25	200	12.5	**1.0**
	Hybrid	37	300	12.3	**1.0**
	Blacklip	61	500	12.2	
Stocking rate	>8.1 kg/m^2	50	100	50.0	**6.2**
	<8.0 kg/m^2	73	900	8.1	

Table 4. Two factor attack rate table for "Happy Abs"

The interpretation of the relative risk in this table is that for the potential risk factor "Species", there appears to be no greater risk of the disease SAD associated with a particular abalone species.

For stocking rate, it appears that the more heavily stocked tanks were at 6.2 times the risk of having SAD than the less heavily stocked tanks (our reference group). This makes biological sense given that increased stocking rate will increase opportunities for spread of infection. Even in the absence of disease, higher stocking rates may be associated with increased stress and mortality on an abalone farm.

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STEP 8

Verify the Diagnosis
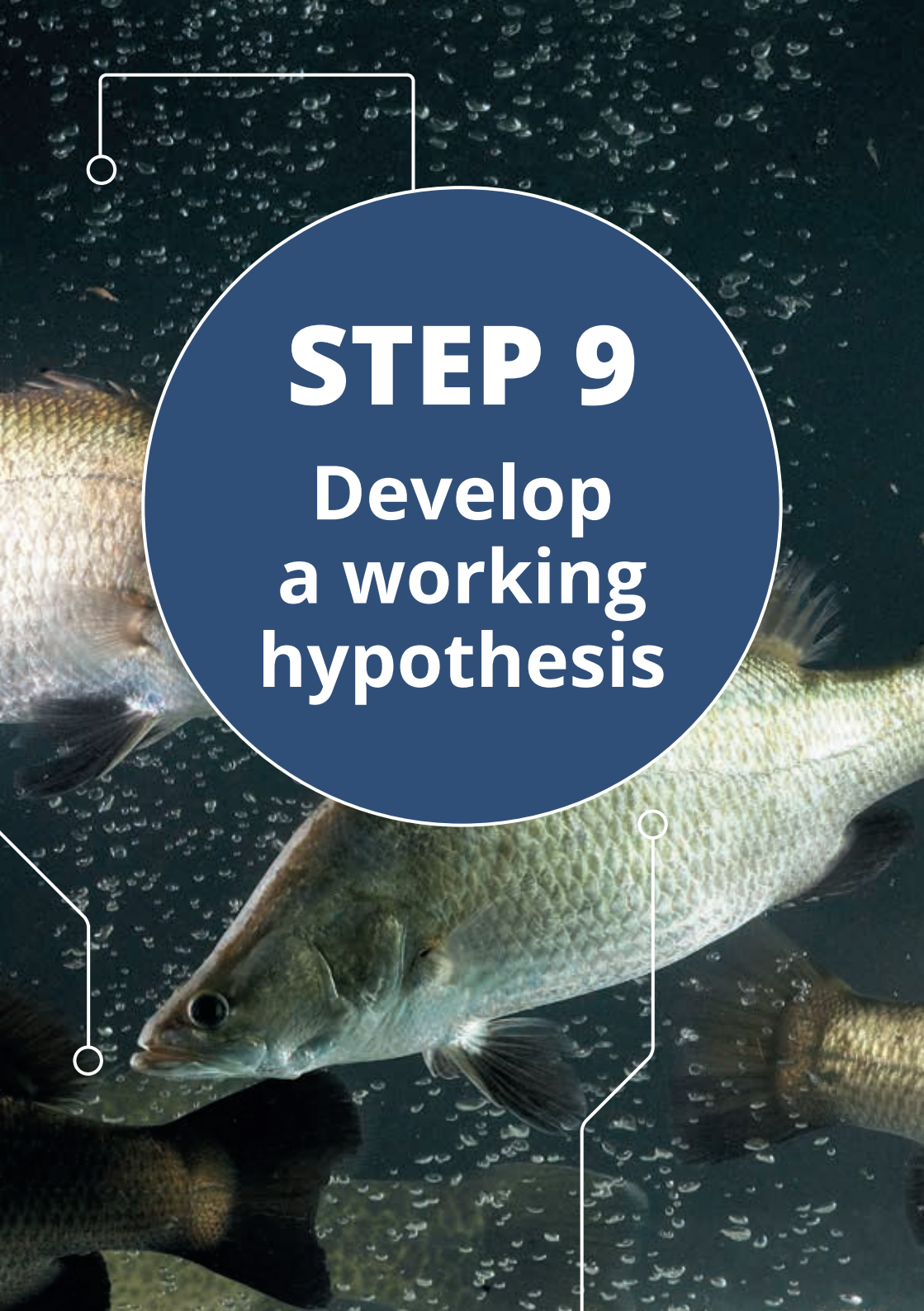
Do we know what is causing the outbreak?

Outbreak investigations can proceed whether we have a laboratory diagnosis or not. Ruling in and out cases based on clinical signs alone may be more difficult but is quite often the starting point of our outbreak investigation.

Remember, we want to know the source of the problem and how to control it irrespective of the cause.

If there are laboratory results available, we can verify the diagnosis by reviewing the test result with the clinical features of the disease including known facts such as incubation period or mode of transmission. Are the results consistent with the findings and biologically plausible? If results are not consistent, is there another disease involved? Are the results correct? Consultation with the laboratory and a review of the results may be required.

A record of cases with symptoms and laboratory findings for each may assist in verifying the diagnosis. This list helps with reviewing our case study at "Happy Abs".

✓ Laboratory result conclusive using a robust diagnostic test (positive result of SAD virus PCR in abalone).

✓ Clinical signs of disease consistent with known information (abalone have classic disease signs of SAD).

✓ Time frames of disease are consistent with known epidemiological information. Time from the suspected introduction of SAD virus onto the farm to when clinical disease is first seen equals the incubation period of the virus (about 3 days).

✓ Transmission event possible (broodstock introduction to susceptible stock in tank M32).

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Photo. Measuring catch (Photo courtesy of Darwin Aquaculture Centre, NT Fisheries)

STEP 9

Develop a working hypothesis

Why did the outbreak occur?

Now is the time to put
it all together.

Following your analysis of spatial, temporal and population patterns it is time to formulate a hypothesis. Sometimes it is useful to think in terms of "who, what, where, when and how" to describe the outbreak. Addressing these questions will help you consider if you have sufficient information and help generate a hypothesis.

From Step 7 there may be some very clear risk factors which can form the basis of control measures to be implemented. Sometimes we have developed preliminary hypotheses which are incorrect. We need to be willing to think flexibly and generate new hypotheses to explain how and why the outbreak occurred.

Once we have a realistic hypothesis that is biologically plausible we are able to undertake corrective actions to slow or prevent the occurrence of a disease. For example, it has been established that the disease bonamiasis in native oysters occurs more commonly in heavily stocked and fouled oyster baskets in autumn.

The control for this hypothesis is to advise farmers to reduce stocking rates and increase basket cleaning during higher risk periods. Ideally hypotheses should be formally tested (but this is beyond the scope of this guideline).

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Photo. Barramundi (Photo courtesy of Mainstream Aquaculture)

CASE STUDY

Returning to our farm "Happy Abs"

In our case study at "Happy Abs" we are fairly confident about the "who, what, where and when". We also know a lot about SAD so implementing controls will be easier than if this was a novel virus. We have established that the infection came onto the farm through translocated broodstock and that more heavily stocked abalone are at greater risk of disease through the attack rate analysis conducted in Step 7. However, how did the SAD virus come to be on the interstate farm "All About Abs"?

Figure 20 is a diagram displaying the hypothesised infection of the SAD virus on "Happy Abs". There were a series of unfortunate circumstances that resulted in the disease outbreak, including illegal movement of infected abalone into the "SAD free zone" outside "All About Abs".

This farm had a level of immunity to the SAD virus through vaccination that masked clinical disease but allowed for subsequent transmission and introduction of virus into "Happy Abs". The SAD virus then became established with resulting clinical signs in the naive population at "Happy Abs".
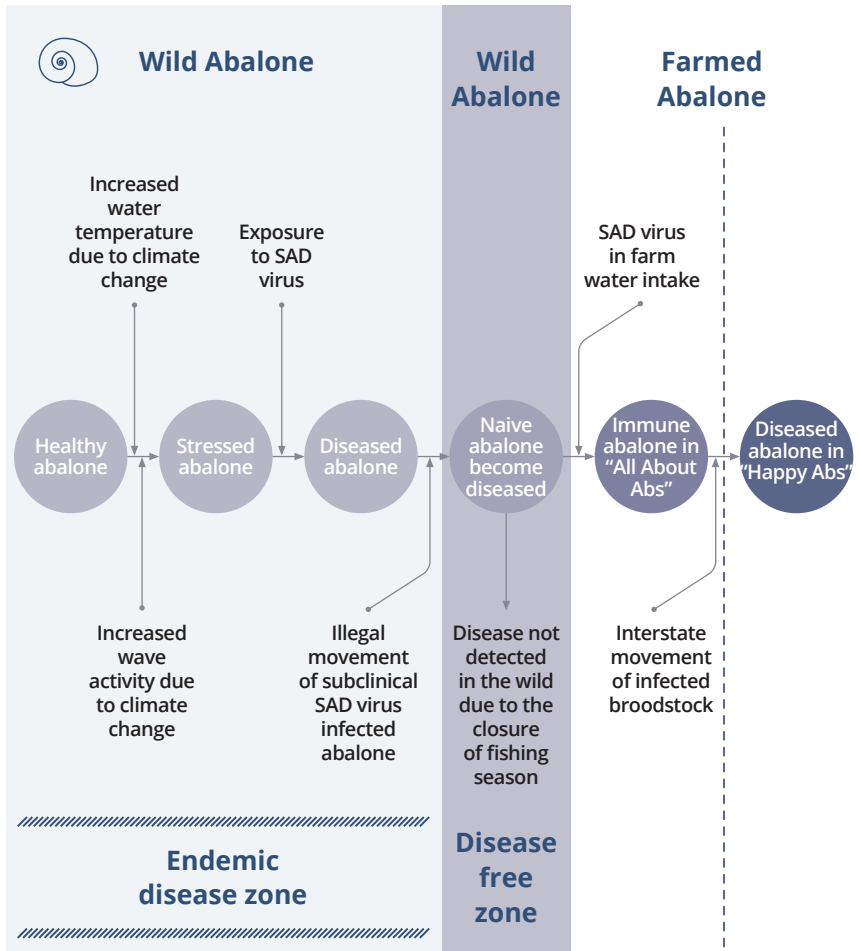
9

Figure 20. Explanatory diagrammatic for SAD virus infection on "Happy Abs"

STEP10

Communicating the findings

Who do we need to tell?

Communicating your findings to the people who need to know is an important task of an investigation.

This should happen not just at the end of the investigation, but also throughout the event with briefings, email updates or formal situation reports. Stakeholder communications are essential to raise awareness and assist with finding new cases. Notifiable diseases must be reported to the relevant state authorities if they are not already involved.

A final report should be prepared following completion of the investigation. This is an opportunity to describe the methods of investigation (what was done), the findings, the conclusions and recommendations.

FINAL REPORTS can be a useful resource for farm management, government agencies, research organisations, industry bodies and other groups. Any reports can potentially be used for legal purposes so should always be accurate and as professional as possible. Information from an outbreak investigation report may assist in adding to the body of knowledge about a disease so consideration should be given to publishing as a case series.

In farm situations, permission should be sought from the farmer prior to distribution to third parties.

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Photo. Pacific oysters (Photo courtesy of Oysters SA)

Support material

Diagnostic test time out

Sample submission

Resources

References

Diagnostic test time out

Diagnostic tests are essential tools that support an outbreak investigation. When you request or run a diagnostic test, do you stop and think about the characteristics and test settings that sit behind the test that you are relying on? We will now briefly look at some of the main factors that influence diagnostic test performance.

Diagnostic sensitivity and specificity

The ability of a diagnostic test to measure what it is intended to, is measured by the test's diagnostic sensitivity and specificity. Note that this is different to analytic sensitivity and specificity.

Diagnostic sensitivity is the proportion of animals with the disease that test positive. A test with high sensitivity will have a low number of false negatives (those with the disease but giving a negative result).

Diagnostic specificity is the proportion of animals without the disease that test negative. A test with high specificity will have a low number of false positives (those without the disease giving a positive result).

Photo. Collie River fish investigation (Photo courtesy of DPIRD, WA)

We often classify diagnostic testing outcomes in a 2x2 table.

	Disease +	Disease –	Totals
Test +	True positives **a**	False positives **b**	Test positives (a+b)
Test –	False negatives **c**	True negatives **d**	Test negatives (c+d)
Totals	Disease positives (a+c)	Disease negatives (b+d)	Total tested **(a+b+c+d)**

Table 5. Diagnostic test sensitivity and specificity 2 x 2 table

In Table 5, the number of appropriate test results are written in each cell. The result is a proportion and is usually expressed as a percentage. It is preferable to calculate confidence intervals around the sensitivity and specificity estimate (refer to the "Resources" section for further information).

Diagnostic sensitivity = true positives/disease positives
= $a/(a + c)$

Diagnostic specificity = true negatives/disease negatives
= $d/(b + d)$

We can't always assume that tests have been validated and that the diagnostic sensitivity and specificity are known. These are features of the test itself and the data may not be available. Test validation requires suitable reference populations of confirmed infected and non-infected animals which may be difficult to access. In the absence of reference populations, comparisons with the performance of other (recognised) tests and/or Bayesian modelling approaches approximate traditional test validation.

Predictive values

We won't know if an animal is truly diseased or not prior to testing. When we have a positive test result is the animal truly positive or just a false positive but unaffected animal? This is where predictive values can be applied.

Positive predictive value (PPV) is the proportion of individuals that test positive that truly have the disease.

Negative predictive value (NPV) is the proportion of individuals that test negative that truly do not have the disease.

Predictive values are very strongly influenced by disease prevalence – as disease prevalence increases, the PPV increases and NPV decreases and vice versa.

Using our 2 x 2 table:
- **Positive predictive value (PPV)** = a/(a+b)
- **Negative predictive value (NPV)** = d/(c+d)
- **Prevalence**   = (a+c)/ (a+b+c+d)

When to use which test

Ideally tests should have both very high sensitivity and specificity but these characteristics are inversely related.

High sensitivity is especially important when:
- There is a high cost of calling a diseased animal negative – you don't want to 'miss' affected animals e.g. screening, quarantine/testing new stock entering a herd.
- Testing for a rare disease (low prevalence).
- Ruling out a disease – you want to be certain about a negative test result.

High specificity is especially important when:
- There is a high cost from calling a non-diseased animal positive (e.g. depopulation based on test results alone, high trade impacts).
- Ruling in a disease – you want to be absolutely certain of a positive result.

Cut-off values

In the real world many tests give results that are on a continuous scale and we need to select a cut off value for positives and negatives. It is always a balance trying to find the appropriate cut-off value as increasing sensitivity will impact specificity and vice versa. Once the cut-off value is set for a particular test, the diagnostic sensitivity and specificity are fixed.

PCR and Ct values

Polymerase chain reaction (PCR) tests are commonly used for detecting diseases, particularly viruses. They work by amplifying the target nucleic acid of a pathogen in a specimen – but they do not distinguish whether the pathogen is dead or alive. In real-time quantitative PCR assays, the amount of DNA is measured after each cycle by a fluorescent signal which is analysed to yield a cycle threshold (Ct) value. This is defined as the number of amplification cycles required to cross a predetermined threshold for the test result to be called positive.

The Ct value is inversely proportional to the amount of target nucleic acid in the sample. For example, a Ct value of 15 would be considered a strong positive and a value of 34 would be a weak positive if the cutoff value was 35. Forty to forty-five amplification cycles is traditionally considered the maximum number of cycles. The threshold is set to distinguish a relevant amplification signal from the background.

The cycle cutoff (designated as 35.19 in Figure 21 below) may be determined for a particular pathogen and test and provides a convenient positive/negative outcome. However, there will be inherent risks of false positives and negatives with this approach.
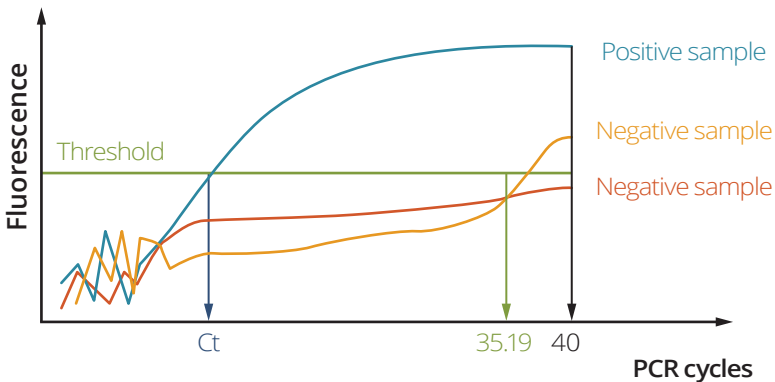

Figure 21. PCR amplification curve and cutoff values
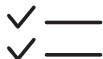
Sample submission

If you suspect an unusual of emergency aquatic animal disease contact the 24 hour national emergency animal disease hotline or your local aquatic animal health professional.

EMERGENCY ANIMAL DISEASE WATCH HOTLINE
1800 675 888

What should be collected in the field?

To complete the laboratory submission form it is ideal if you know the following:

- Owner and property details.
- Location, including GPS coordinates where disease is occurring in a wild population.
- Species, age, size.
- Number of deaths, number sick, total number on the farm and number examined.
- Description of the outbreak, when it began, clinical signs and behaviour.
- Type of farming system.
- Vaccinations, treatments and feeding systems.
- Recent introductions, recent management changes or stressful events.
- Water and environmental data.
- Lesions and necropsy findings (including findings from examining gill snips or skin scrapes in the field).

Photographs

- Sick and dead animals.
- Lesions, abnormal internal organs.

Samples

- Ideally collect both fixed and fresh samples.
- From affected and healthy animals at least five per group, if possible, in consultation with laboratory staff.
- If more than one water source is used for production, affected animals from each water source should be collected.
- Only pool samples where advice has been sought from the laboratory and animals are from the same population group.
- Take samples aseptically (use sterile containers and gloves) where possible.
- Describe lesions and take measurements.
- If trying to collect a representative sample from a tank or pond, attempt to select animals from a range of sites.
- See sample collection guides in Table 6.

Diagnostic Procedure	Method of submission				
	Live	Freshly killed on ice	Frozen	Preserved in 95% ethanol/ other nucleic acid stabiliser	Preserved in formalin
Toxicology	YES	YES	YES		
Parasitology	YES	YES	Limited		Limited
Bacteriology	YES	YES			
Virus isolation	YES	YES			
Molecular (e.g. PCR)	YES	YES	YES	YES	
Pathology · Gross examination · Histopathology · Immunohistochemical staining	YES	YES			YES

Table 6. What type of sample is used for what test?

What does all this mean?

- Toxicology is looking for contaminants in the tissues that might have caused the disease and is very specific.
- Parasitology is looking with a microscope for internal and external parasites or eggs.
- Bacteriology is the culture of tissue to grow and isolate bacteria for identification under the microscopic with the use of special stains.
- Virus isolation is the gold standard diagnostic method to identify viruses. It involves attempting to grow the virus in cell lines to amplify the virus in number. Further tests may then be performed to identify the isolated virus.
- Molecular tests such as polymerase chain reaction (PCR) amplify and detect viral genetic material in tissue.
- Gross examination is looking at the external appearance of the animal or an organ.
- Histopathology evaluates the effect of the disease in tissues at a microscopic level.
- Immunohistochemistry is sometimes used to look for the disease agent in the tissues.

Diagnostic hot tips – gill clips and skin scrapes

Parasites are often implicated in poor performance or death in aquatic animals. However, parasites may be lost during transit, particularly if animals are anaesthetised or frozen, or there is a delay in getting live animals to the lab. Accurate parasitology is all about timeliness.

Examining fresh gill clips or skin scrapes off a very recently euthanised fish gives the greatest chance of seeing motile parasites with a light or dissecting microscope. Some farms and aquatic animal health practitioners have access to such equipment which makes this task relatively straightforward. Smearing affected material (gill filaments or skin scraping) on a microscope slide then placing a cover slip over the material will allow easy visualisation.

Parasites can be fixed from smears or scrapes but this may require specialised fixatives. Gills and skin segments that have been preserved in the standard fashion for histopathology will be diagnostic for parasites if this is done promptly following euthanasia, though identification of parasites is much less accurate.

Photo. Salmon gills (Photo courtesy of Phillipa Sims, NRE Tasmania)

Sampling for Histopathology

Step 1. Sample collection

	Finfish	Crustacea	Mollusc
Larvae/post larvae/fry	Place 25 – 50 or more directly into the fixative.	Place 25 – 50 or more directly into the fixative.	Place 25 – 50 or more directly into the fixative.
Juveniles	For fish <4cm long – cut off the gill opercula. Cut open the abdominal cavity, pull internal organs out slightly to expose. Place in fixative.	For crustacea 10 – 30mm long cut between the head and the tail and place both pieces in fixative.	Up to 10cm length remove the soft tissues (as a group) from each shell, cut into the middle of these tissues and place in fixative.
Adults	For fish >4cm dissect 0.5 to 1cm sections for each organ (take whole organ if <1cm thick). Collect the following: gills, kidney, liver, spleen, heart, skin, muscle, gut, gonads and brain.	Over 30mm long. Inject 1 – 2 ml fixative into the head and 1 – 2 ml into the tail. Remove head and a section of the tail and place in fixative.	If the adult is larger than 10cm in length and 1cm thick, cut into sections to improve preservation.
Large adults	As above.	Excise the organs of interest and place in fixative.	As above.

Table 7. Sample types to collect for histopathology

Ensure samples are representative of the lesions:

- Sample the interface with normal tissue.
- Sample areas of different colour or consistency.
- Sample size should be no more than one centimetre x one centimetre.
- Consider multiple samples for large lesions.
- The below diagram (Figure 22) represents the collection of samples for both histology and culture, virus isolation or molecular tests.
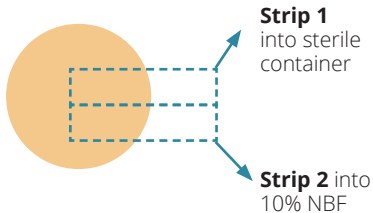
Lesion sampling

Guide: cut 0.5 – 1 cm wide and deep

For large Lesions

Aerial view

Strip 1 into sterile container

Strip 2 into 10% NBF

For small Lesions

Aerial view

Strip 1 into sterile container

Strip 2 into 10% NBF
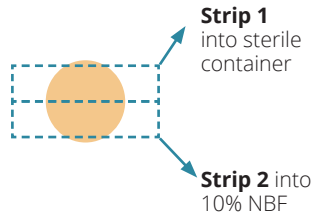
---------------- **Section cut lines**

Figure 22. How to best sample a lesion

Step 2. Fixation

The fixative to use for histopathology is usually 10% neutral buffered formalin (NBF).

To make up one litre of 10% NBF

100 millilitres formalin (37 – 40% w/v formaldehyde solution)
+ 900 millilitres saline solution.[5]

Note: Formalin can be purchased from chemical supply companies and some rural stores. Most veterinary surgeries, pathology laboratories and hospitals will have supplies. Saline solution can be purchased from pharmacies.

- Immerse tissue sections or small <1 centimetre whole animals in 10% NBF fixative at a 1:10 ratio. Don't overfill the container (Figure 23).
- Store at room temperature away from the sun.

Figure 23. Incorrect and correct proportion of tissue to fixative (Photo courtesy of DPIRD, Western Australian Fish Kill Program)

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5 Replace saline solution with sea water for marine species.

Methods of Submission of Samples

Specimen	How to package	How to transport
Live animal	**Finfish and prawn:** Sealed plastic bag partially filled with water containing air or charged with oxygen. Allow 2L of water per 5 – 10 animals (up to 10cm length) filling bag to ¼ full.	Strong polystyrene cold box with plastic liner. Keep at appropriate temperature, if warm weather include small amount of ice in separate bag for non-tropical species.
	Mollusc: Wrap in damp paper towel or cloth and fill bag with air or oxygen if available.	
	Crayfish: Use rolled up damp newspaper or mesh onion bags as packing material to cushion.	
Freshly killed animal on ice	Individually wrapped and sealed in plastic bags (with identification).	Strong polystyrene cold box with plastic liner. Generous amounts of ice in separated plastic bags.
Frozen animal	Individually wrapped and sealed in plastic bags (with identification).	Strong polystyrene cold box with plastic liner. For some toxicological tests, maintain samples frozen by packing with dry ice in separate plastic bags.
Preserved specimens	Ensure that there is 10 times the volume of fixative for each volume of tissue. Fix for at least 24 hours. Samples can be sent in fixative in a leak-proof container if postage regulations allow. Alternatively pour off the fixative and send specimens in sealed container with paper towel soaked in preservative.	Strong container lined with plastic.

Table 8. Packaging and transporting a range of specimens

Water samples

Check online for up-to-date detailed guidelines and Australian standards for collection and analysis of water samples (www.waterquality.gov.au/anz-guidelines). Methodologies will vary between laboratories so it is best to contact the lab before collection, they may be able to provide appropriate containers.

Some tests such as oxygen and pH are best conducted onsite and may require specialised equipment. Other tests such as ammonium and nitrate/nitrite must be analysed rapidly or samples frozen. Samples should be kept cooled and protected from the light.

Care should be taken in collecting sterile samples for bacteriological analysis, including ensuring the sampling container is sterile.

If you are attending a wild aquatic animal outbreak, collect three water samples:
1. at the kill site
2. upstream and
3. downstream.

Resources

Epidemiology resources

Centers for Disease Control and Prevention. Using an epi curve to determine most likely period of exposure. www.cdc.gov/training/quicklearns/ exposure/

Department of Agriculture, Fisheries and Forestry. (2007). National Investigation and Reporting Protocol for Fish Kills. Commonwealth of Australia. www.agriculture.gov.au/sites/default/files/ sitecollectiondocuments/animal-plant/aquatic/field-guide/4th-edition/amphibians/fish-kill-protocol.pdf

Lilley, J., Callinan, R., Chinabut, S., Kanchanakhan S., MacRae, I. and Phillips, M. (1998). Epizootic Ulcerative Syndrome (EUS) Technical Handbook.

Sergeant, E. and Perkins, N. (2015),
Epidemiology for Field Veterinarians: An Introduction.
CAB International.

Thrusfield, M. (2018),
Veterinary Epidemiology 4th Edition.
John Wiley and Sons Ltd.

Sample submission resources

Delamare-Deboutteville J, Khor L, Ali S and Mohan CV. (2021) Aquatic Animal Health Package of Practices: Fish sampling for disease diagnostics. Penang, Malaysia: WorldFish. Guidance Note. 211fa198eabfb31714dd51620b0a4231.pdf (worldfishcenter.org) (accessed 21/9/2022)

Department of Primary Industries and Regional Development. Disease diagnostic service. www.fish.wa.gov.au/Sustainability-and-Environment/Fisheries-Science/Aquatic-Animal-Health/Pages/Disease-Diagnostic-Service.aspx (accessed 21/9/2022)

Handlinger, J. (2008) Finfish sampling. www.agriculture.gov.au/agriculture-land/animal/health/laboratories/procedures/anzsdp/finfish-sampling (accessed 21/9/2022)

Queensland Government. Submitting fin fish for lab examination. www.business.qld.gov.au/industries/farms-fishing-forestry/fisheries/aquaculture/managing-disease/fin-fish-examination (accessed 21/9/2022)

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Sergeant, E. (2012) Introduction to epidemiology and disease outbreak investigation. Fisheries Research and Development Corporation Project 2009 – 315.

Southwood, t. (1978) Ecological methods with particular reference to the study of insect populations. 2nd Edition. Chapman and Hall, London.