Travel to field site Introduction to larval collections

P T Joshi M. Sc. FISCD
Former State Entomologist and Consultant Sate Entomologist NVBDCP&IDSP Commissionerate of Health ,Medical Services & Medical Research & Education,
Former Consultant Health Environmental Sub-Group of Sardar Sarovar & Narmada Nigam (SSNNL) Govt of Gujarat - Gandhinagar

Know-Mosquito Larvae

Mosquito larvae, called “wrigglers,” are aquatic, with a large head and thorax and narrow, wormlike abdomen; they typically hang just below the water surface, breathing air through tubes at the end of the abdomen. When disturbed, they wriggle downward.

The pupae, called “tumblers,” are curled like a comma and also hang just under the water surface, breathing through air tubes.

Mosquito larvae are surprisingly complex, with a sophisticated sense of smell that enables them to find food, avoid predators and thus become healthy adult mosquitoes with greater ability to transmit disease to humans.

While it was previously known that water-dwelling mosquito larvae can taste chemicals in the water, this research is the first to show that they can also stick their antennae just above the water’s surface and detect odours in the air.

Mosquito larvae are surprisingly complex

Sampling methods : Mosquitoes & its Aquatics Stages

Sampling methods for mosquitoes can be divided into four main categories:

Methods for collecting flying adults,

Resting adults,

Larvae and eggs.

The combined use of these methods depends on the objective of the surveillance or monitoring campaign, the target species, the environmental conditions at the selected sampling sites and the availability of resources

Selection of sampling methods

- The selected sampling method is chosen according target species, life stage and status.
- Sampling adult flying mosquitoes is the preferred method when all immatures have emerged when sampling larvae is more time consuming or when breading sites are scarce because of dry conditions.
- Some species are difficult to collect with the available adult traps and should therefore be sampled in the larval stage.
- Larval sampling may also be required for vector control purposes: in case of mosquito nuisance, e.g. it is essential to detect the source of the problem, so identification of the larval breeding sites of the species is key.

Study design: Cross-sectional and Longitudinal survey

- The study design should meet the main objectives:
- A cross-sectional design is recommended primarily for distribution and abundance assessments;
- A longitudinal design is recommended for the collection of data on population dynamics.
- Cross-sectional surveys collect data to assess the presence/absence or vectorial capacity (field components) of the target species at randomly selected or risk-based study sites and over a short time period.
- Cross-sectional surveys may be repeated periodically.
- Usually, in a repeated cross-sectional survey, sites sampled at one point in time are not intentionally sampled again, although a site of a previous survey could be randomly selected for a subsequent one.
- However, identical sites can be intentionally re-sampled: hotspots of invasive species, for example, are often sampled several times a year.

Cross-sectional and Longitudinal survey

- Longitudinal surveys are research studies that involve repeated sampling of the same sites.
- A limited number of sites selected by a predefined geographical range, biotope type/s and time frame are monitored over one or more mosquito seasons.
- These surveys could be used to assess vector presence/absence and population dynamics (e.g. seasonality, density peaks, number of generations) and vector abundance.
- Density peak assessments are essential to depict time windows for abundance sampling.
- If combined, both types of data can later be used risk assessment.
- Data on presence/absence can be collected by a number of appropriate sampling methods but the estimation of population dynamics and abundance should be carried out by a single sampling method in accordance with a standardized protocol.

Planning of Sampling:

- When planning sampling activities, it is important to obtain land cover maps of the area of interest and its surroundings.
- Basic ecological and meteorological knowledge of the sampling area is important for the success of the study.
- However, this is not possible in all areas and should not be a limiting factor when planning sampling activities.
- There are a number of tools available that facilitate the planning of sampling activities based on state-of-the art geographic information systems and integrated software components.
- They allow for the rapid and cost-efficient planning of surveys, which in turn increases the overall success rate.

Selection of study sites and exact location of sampling sites

- Study sites can be selected based using the following criteria:
- Identified gaps in the knowledge of the distribution of the species
- The geographical area to be prospected is identified based on the following factors:
- There are no field data or existing data are incomplete and/or outdated
- Data collection through passive surveillance is impossible
- Theoretical species distribution models highlighting areas with high probability of presence where model validation with field data is required.
- Once the study sites are identified, the exact locations of all sampling sites have to be determined.
- This can be done using online satellite imagery (e.g. Google Earth).
- These maps can be used to identify urban, suburban and rural zones; floodplains and rice fields; urban ‘green and blue islands’; large animal sheds and bunkers or caves.
- Maps also permit to identify resting vegetation (groves, forests) in or nearby flood lands.

Selection of study sites and exact location of sampling sites: Flying Adults

- Which locations are selected for sampling also depends on sampling methods:
- Flying adults. Identify places which meet the following criteria:
- Appropriate location and good accessibility
- Moist and protected from wind (mosquitoes prefer to fly through bushes and shrubs, and try to avoid open terrain like meadows; hedges are often used as flight corridors
- Out of public sight and not accessible by children.
- Resting adults. Identify all types of animal shelters and all natural and artificial hiding places.

Selection of study sites and exact location of sampling sites: Larvae

- In natural areas, larvae can be found in e.g. puddles, road tracks, swamp areas, drains, ditches, irrigated croplands, streams, riverbeds, ponds, and tree holes, but also in human-made containers such as drinking troughs, discarded waste containers, tyres, and tarpaulins.
- In urbanised areas, larval sampling should focus on the available human-made water bodies (both in private and public areas), below and above ground level (e.g. discarded containers, flower vases, flower pots in gardens and cemeteries, used tyres left outdoors, rain water barrels, road drains and catch basins, pits).
- Eggs. Identify places close to or under vegetation or near buildings. The position of all ovitraps should be marked on a map.

Conservation of specimens

Larvae

- **For immediate morphological or genetic identification**:
- collection in vials with 70–80% ethanol.
- **For morphological identification only after further development:**
- collection in vials/small containers together with water from breeding place for rearing L1-L3 larvae to L4 larvae (which can be identified with higher reliability) or for keeping the larvae until adult emergence.

Conservation of specimens

Eggs

Oviposition supports can be stored in a closed plastic bag, at room temperature or in a fridge (5–15 °C). The bag should not contain free water, but should still be humid (around 55% relative humidity).

For genetic identification (PCR, DNA sequencing), the eggs can be put in 70–80% ethanol.

For MALDI-TOF MS, eggs are best kept on the oviposition support (but should not dry out) and transferred to (dry) microtubes before sending them out for analysis.

Identification methods

- Eggs
- Eggs can be recognised by their general shape, their size and the structure of the egg shell (exochorion). However, the morphological identification of eggs can be challenging, and identification keys only exist for a few species.
- Eggs can be flooded in the laboratory to let the larvae hatch and identify the resulting 3rd or 4th larvae instars or adults, although this can be inefficient for some species, in particular in case of diapausing eggs.
- MALDI-TOF MS or molecular methods are useful methods for accurate identification.

Table. Checklist for mosquito field specimen collections

Field data and parameters	Flying adult collection	Resting adult collection	Larval sampling	Ovitraps
Traps				
Checked and functioning traps	X			
Charged batteries	X			
Collecting nets	X			
Dry ice containers (part of the trap) or CO_2 gas bottles and release system	X			
If dry ice is used: dry ice in an insulated box	X			
Ladle/scoop for dry ice	X			
Insulated box for transport of collected nets	X			
Mechanical aspirator (BioQuip insect vaccuum, Hausherr's handheld aspirator, or own construction) or mouth aspirator	X	X		
Hand net		X		
Back pack aspirator (only if available on site)		X		
Dipper			X	
Fine-mesh aquatic net			X	
Fine-mesh sieve			X	
Kitchen ladle			X	
Pipettes			X	
Ziplock plastic bags (10x15 cm)				X
Ovitraps: black plastic bowls (diameter 11 cm, height 9 cm, volume 0.62 l), piece of polystyrene (5 x 5 x 2.5 cm)				X
General				
Rope	X			
Swiss army knife	X			
Permanent marker	X			
Heavy-duty adhesive tape	X			

Field data and parameters	Flying adult collection	Resting adult collection	Larval sampling	Ovitraps
String	X			
Meteorological data logger	X	X		
Electric torch	X	X		
Labels	X	X	X	X[2]
Pencil	X	X	X	X
GPS or smartphone with geolocation function	X	X	X	X
Vials		X	X	X[1]
Ethanol, 70%[1]		X	X	X[2]
Magnifying glass		X	X	X
Entomological forceps		X		X[2]
Ethyl acetate		X		
Insect boxes		X		
Insect pins (n°2), micro pins, and small polyethylene foam or cardboard pieces		X		
Tubes			X	
Tissue paper				X
Outline map				X

Sampling…..!! What time of year?

Sampling should be performed during the mosquito season (April–October in temperate regions).

Samples can be collected throughout the year as long as the mean daily temperature remains above 10 °C.

To collect data on the initial population and their different overwintering locations, collect the first samples during the first week of the mosquito season when overwintering females start searching for blood meals.

The first week of the mosquito season usually coincides with the short period when the outside temperature surpasses the temperature inside the overwintering shelters (10–12 °C).

In addition to sampling at the beginning of the season, samples should be collected around the seasonal peak period (plus/minus one week) to capture peak abundance data.

Sampling….!! What time of day?

Samples can be collected all day and night, assuming that most battery-powered traps run for 24 hours; collection should start no later than in the early afternoon and continue into late morning the next day in order to catch diurnal biters; a more precise schedule should be applied for abundance monitoring (see below).

If sample collection is targeted to a particular species in order to save time, make sure that the peak of biting activity of that species is covered.

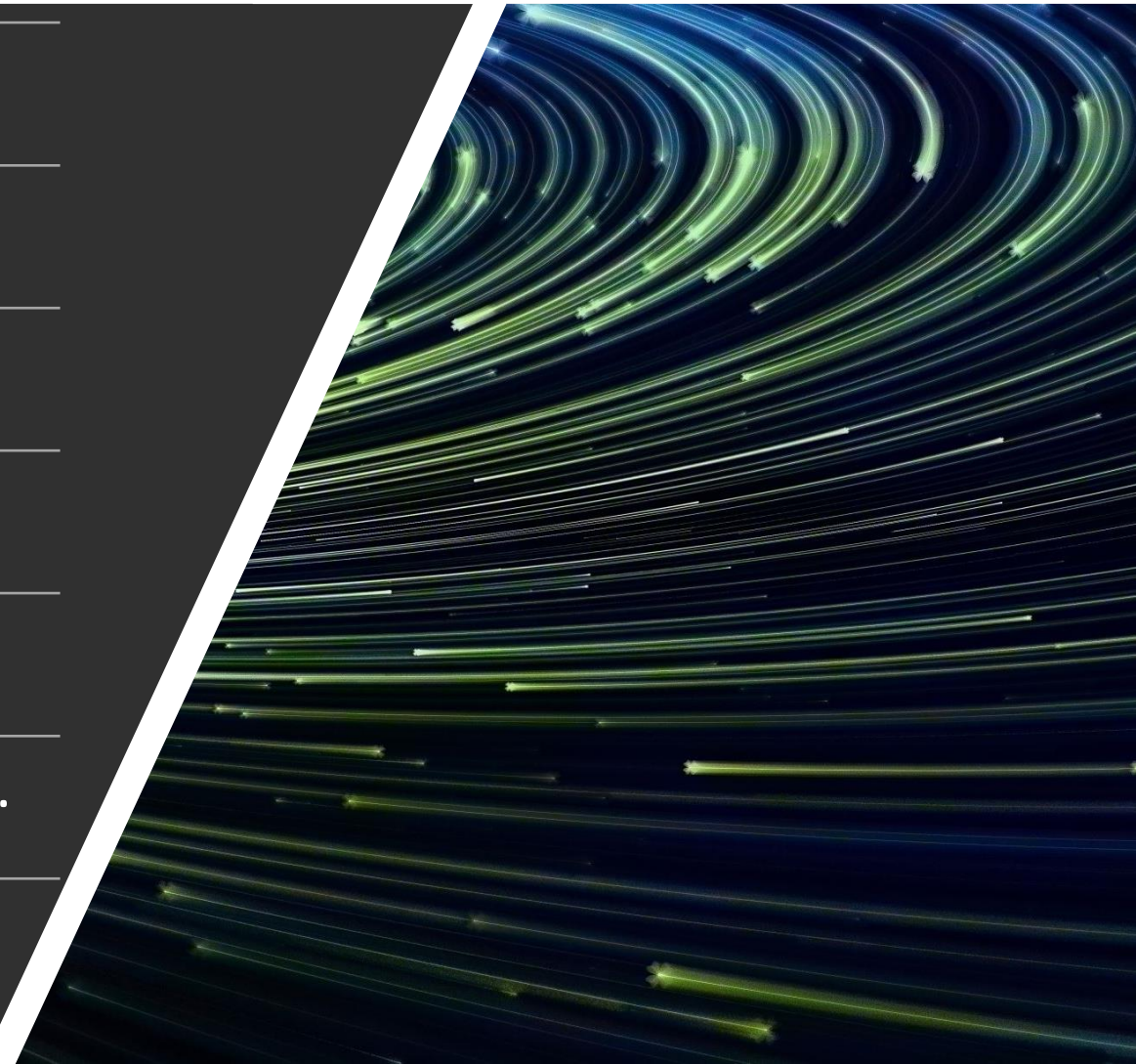
For each study area, the local literature (or data published for neighbouring countries or the same latitude) about seasonal activity of the targeted species (e.g. start of activity of hibernating females and seasonal peak) should be searched to find the best times for sample collection.

Sampling Methods : Larvae

Trapping methods

Six main classes of larval habitats can be distinguished:

- Stagnant temporary water bodies (ditches, ponds, forest ditches, fens, flooded meadows or forests)
- Running waters (rivers, streams, ditches, drains)
- (Semi-)permanent water bodies with vegetation (ponds with vegetation, marshes, canals)
- Semi-natural water bodies without vegetation (e.g. puddles, road tracks, new ditches)
- Natural containers (e.g. tree holes, rock pools)



Sampling Methods

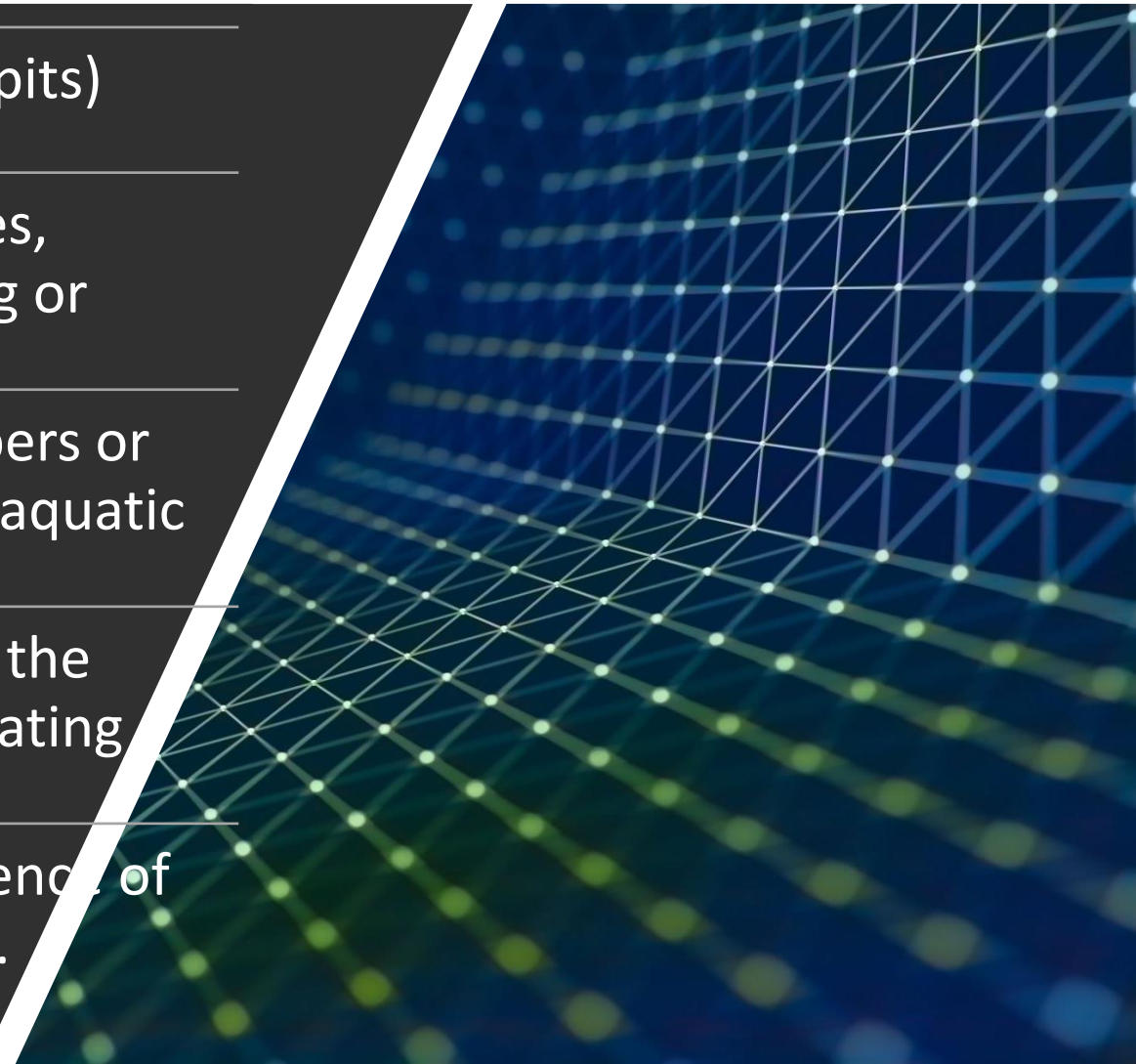
Man-made (artificial) containers (pots, catch basins, pits)

Depending on the size and type of their breeding sites, mosquito larvae can be collected by : netting, dipping or aspirating.

Larger water bodies can be sampled with classic dippers or plastic trays (a frisbee) or a fine- meshed (≤ 0.5 mm) aquatic net (aquarium net) and sieve.

Smaller water bodies (tree holes) can be checked for the presence of larvae by dipping with a ladle or by aspirating water with a tube or a pipette.

Collected water can be inspected better for the presence of juveniles when decanted in a white plastic tray/bowl.

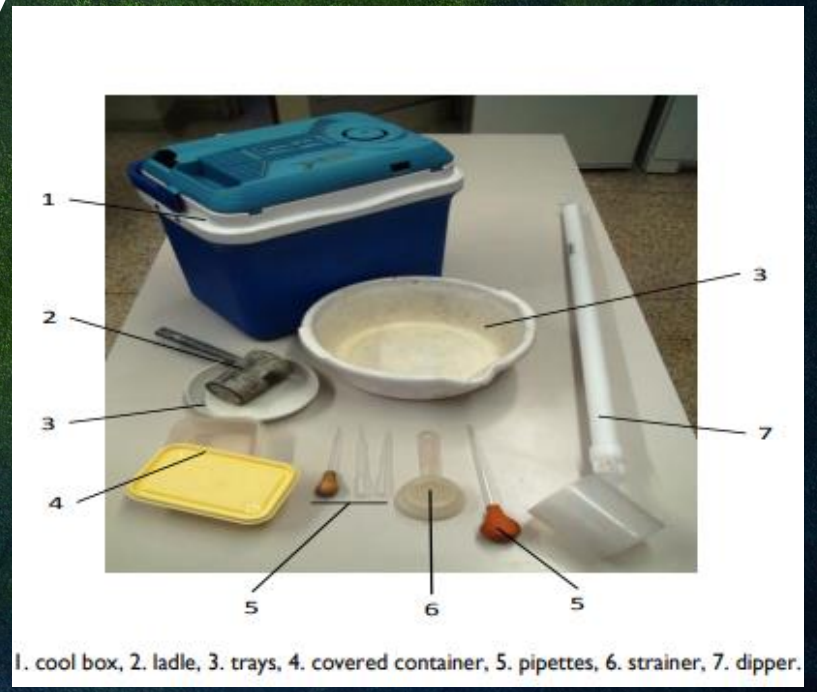


Sampling Methods

Dippers can be used as a survey tool to determine the abundance of larvae by taking several samples from designated sites in the habitat of interest and then counting the larvae of each dip.

Netting, which allows to sample larger parts of the habitat, is more appropriate to determine presence/absence.

The netting/dipping method will vary with water depth, presence of aquatic vegetation or debris, and water clarity.



1. cool box, 2. ladle, 3. trays, 4. covered container, 5. pipettes, 6. strainer, 7. dipper.

Sampling Methods

Five to 20 sweeps/dips are recommended, depending on the site size and diversity of micro habitats (with or without vegetation, floating or erect vegetation, different water depths, shaded or sun-exposed).

Several dipping techniques exist, with different efficiency to collect the various mosquito genera (Table).

Complete or partial submersion (depending on the water level) is recommended for routine immature monitoring of all species (general, non-genus-specific approach) in order to make comparisons across a range of aquatic habitats

Dipping techniques

Dipping method	Targeted mosquito genera	Method details	Notes
Shallow skim	*Anopheles*	The leading edge of the dipper is submerged at approximately 45° and about 2.5 cm below the water surface. The dipper is drawn along the water surface and filled at the end of the stroke.	The method works better for *Anopheles* larvae that remain at the water surface longer than other mosquito larvae after the dipper enters the water. This is a good sampling technique when submergent macrophytes (aquatic plants) have leaves just below the water surface.
Complete submersion	*Aedes*, *Ochlerotatus*, (*Culex*, *Culiseta*)	The dipper is submerged quickly in open water, usually in floodwater habitats. The dipper is brought up to the water surface through the submerging larvae that have reacted to the disturbance created by submerging the dipper.	This method is used primarily to sample mosquitoes whose larvae react rapidly to the dipper entering the water, but are visible. It is also appropriate for sampling larvae adjacent to vegetation. The dipper is brought to the water surface while brushing against the emergent vegetation.
Partial submersion	*Anopheles*, *Culex*, *Culiseta*	The dipper is submerged at approx. 45° along the emergent vegetation. Water flows rapidly into the dipper. The dipper should not be moved horizontally but vertically to scrape along the edge of the emergent vegetation.	The method works well when sampling in robust emergent vegetation such as cattail and bulrush. The suction created by water flow into the dipper and scraping also collects small insect predators and herbivores associated with mosquito larvae on or near the vegetation.
Flow-in	*Aedes*, *Ochlerotatus*, (*Culex*)	This technique is used in shallow water that has a depth < height of the ladle of the dipper. The bottom of the dipper is pushed into the substrate and the water with associated larvae and debris flow into the dipper.	This method works well in shallow habitats, root masses and other habitats that are shallower than the dipper's profile.
Scraping	*Coquillettidia*	The dipper is scraped against the underside of floating vegetation to dislodge attached larvae. The scraping action is usually a vigorous back-and-forth motion.	Used to sample larvae that reside under and usually attached to floating vegetation or the roots of floating plants. Because a vigorous back-and-forth motion is used with the dipper completely submerged, this technique works best with dippers having a screened bottom.
Simple ladle	*Culex*	A quick flip of the wrist is used to completely submerge the dipper just below the water surface.	Not a preferred method, especially if the sample is not taken adjacent to a mosquito microhabitat. This technique

Sampling Methods…Dipping

- Larvae should first be transferred with a pipette to a small cup or bowl with fresh clean water as a washing procedure.
- If much debris or sediment is still present, additional serial transfers should be made until all suspended particles are eliminated.
- Using a pipette, as much water as possible should be removed from the cup or bowl.
- In the laboratory, water should be heated to about 60 °C and poured into the cup or bowl.
- In the field, the larvae should first be transferred to a vial with a pipette and then pure ethanol should be added.

Sampling Methods…Dipping

- As soon as the larvae float up to the surface, the liquid is removed with the pipette and replaced with 70–80% ethanol.
- After five minutes, the larvae are transferred with the pipette (do not use forceps) to a vial with 70–80% ethanol.
- After filling the vial completely with ethanol to remove all air, it should be capped tightly.
- No more than 20 larvae should be placed in a 50 ml single vial because the water contained in the bodies of the larvae will significantly dilute the concentration of ethanol and jeopardise preservation.

Sampling Methods…Eggs

- Sampling methods: Trapping methods
- Ovitraps can be any kind of black plastic bowls (0.3 to 2 l volume) filled with water (ca. 2/3) and supplemented with an oviposition support (e.g. a wooden stick or a piece of polystyrene).
- The size of the ovitrap (i.e. the volume of water it can contain) has to be adjusted to the trap-checking frequency as well as to the local rainfall frequency and intensity in order to prevent the trap from drying out.

Specific guidelines for sample collection

Recommendations for trap density, frequency of trapping and trapping period are listed in Table 4.

Table 4. Recommendation for trap density and period of trapping

Surveillance aim and sites	Density of traps	Frequency of trapping	Period of trapping
Introductions at point of entry			
Main parking lots at country borders[1], highways and road axes that originate in colonised areas, storage platforms of imported tyres	1/2500 m²	biweekly	Apr – Nov
Ports	1/5000 m²	biweekly	Apr – Nov
Airports	1/ha	monthly	Apr – Nov
Persistence in colonised area			
Inspection of colonised area	1/5 ha	biweekly	Apr – Nov
Abundance and seasonal dynamics	6/site	biweekly	Jan – Dec[2]
Spread into areas surrounding colonised area			
Inspection around colonised areas	1/15 ha	monthly	Apr – Nov

Source: Adapted from [1]

[1] Includes parking lots at commercial centres close to country borders.

[2] Required during the first year; can later be limited to the period of development in the local climate.

Identification methods : Eggs

Eggs can be recognised by their general shape, their size and the structure of the egg shell (exochorion). However, the morphological identification of eggs can be challenging, and identification keys only exist for a few species.

Eggs can be flooded in the laboratory to let the larvae hatch and identify the resulting 3rd or 4th larvae instars or adults, although this can be inefficient for some species, in particular in case of diapausing eggs.

MALDI-TOF MS or molecular methods are useful methods for accurate identification.

Quality control

- To ascertain the quality of the identifications, 10% of the collected samples can be verified by an external expert, either by using morphological or non-morphological/ molecular identification techniques.


THANKS