Australian Government
Department of Sustainability, Environment, Water, Population and Communities

JCU JAMES COOK UNIVERSITY

A REPORT FOR THE AUSTRALIAN GOVERNMENT DEPARTMENT OF SUSTAINABILITY, ENVIRONMENT, WATER, POPULATION AND COMMUNITIES

# Hygiene protocols for the control of diseases in Australian frogs

**June 2011**


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The authors gratefully acknowledge:

Chris Banks, Dr John Clulow, Dr Graeme Gillespie, Professor Rick Speare and Russel Traher,for their contributions to components of the original project resulting in the production of this final report.

Cover photo: *Taudactylus eungellensis* – Eungella day frog. K. Murray



Funding for this project (Procurement Reference Number: 1011-1151) was provided by the Australian Government Department for Sustainability, Environment, Water, Population and Communities. This project progresses the implementation of the *Threat abatement plan for infection of amphibians with chytrid fungus resulting in chytridiomycosis* (Commonwealth of Australia, 2006).

This report should be cited as:

> Murray, K., Skerratt, L., Marantelli, G., Berger, L., Hunter, D., Mahony, M. and Hines, H. 2011. Hygiene protocols for the control of diseases in Australian frogs. A report for the Australian Government Department of Sustainability, Environment, Water, Population and Communities.

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**Hygiene protocols for the control of diseases in Australian frogs**

## 1. Who should use this document?

- This protocol is intended for use nationally by conservation agencies, zoos, scientific research staff, industry organisations (e.g., the pet industry), wildlife consultants, fauna surveyors, students, frog keepers, wildlife rescue and carer groups, frog interest groups/societies and other key interest groups who regularly deal with or are likely to encounter frogs.

- This protocol outlines the expectations of the Department of Sustainability, Environment, Water, Population and Communities (DSEWPaC) regarding precautionary procedures to be employed when working with frogs in Australia. The protocols were developed in collaboration with recognised experts in the fields of wildlife health, husbandry, research and conservation. The intention is to promote implementation of hygiene procedures by all individuals working with Australian amphibians.

- DSEWPaC recognises that some variation from the protocol may be appropriate for particular research and frog handling activities. Such variation should accompany any licence applications or renewals submitted to the relevant regulatory bodies for independent consideration. Variations should follow a risk analysis process which broadly involves hazard identification, risk assessment, risk management and risk communication.

Where *ex-situ* activities are proposed, these guidelines should be used in conjunction with the "**Guidelines for captive breeding, raising and restocking programs for Australian frogs**", which can be found here: http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11**.**

## 2. Objectives

The objectives of the hygiene protocols are to:

- Improve the control of diseases in Australian frogs
- **Improve preparedness for an emergency response** to new amphibian disease incursions in Australia
- **Recommend best-practice procedures** for personnel, researchers, consultants and other frog enthusiasts or individuals who handle frogs
- **Suggest workable strategies** for those regularly working or considering working in the field with frogs or where frogs may exist
- **Provide background information** and guidance to people who provide advice or supervise frog related activities
- **Inform regulatory bodies and animal care and ethics committees** for their consideration when granting permit approvals

## 3. Introduction

Amphibians have declined globally. In the first global amphibian assessment, at least 43% of amphibian species with sufficient data were found to have declined in recent decades, 34 species were extinct and a further 88 were possibly extinct (Stuart et al. 2004). In 2010, approximately 30% of amphibians were threatened globally (http://www.iucnredlist.org/documents/summarystatistics/2010_4RL_Stats_Table_1.pdf).

Diseases are responsible for many amphibian declines and extinctions and their risk needs to be addressed. Laurance et al. (1996) first proposed the 'epidemic disease hypothesis' to account for Australian amphibian declines. Shortly after, an unknown chytridiomycete fungus was seen infecting the skin of sick and dying frogs collected from montane rain-forests in Queensland and Panama during mass mortality events associated with significant population declines (Berger et al. 1998; Longcore et al. 1999). The fungus was subsequently found to be highly pathogenic to amphibians in laboratory trials by inducing development of skin pathology, morbidity and mortality similar to that seen in the wild frogs. The disease was called chytridiomycosis and the fungus described as a new species *Batrachochytrium dendrobatidis* (Bd), also known as the amphibian chytrid fungus.

Bd has been found infecting over 350 species in two amphibian orders (Anura and Caudata) from all continents where amphibians occur (http://www.bd-maps.net/). Sixty-three (~28%) of Australia's 223 (as listed by IUCN 2008) amphibian species are now known to be wild hosts for Bd (Murray et al. 2010a; Murray et al. 2010b), and over half of Australia's species may be naturally susceptible to Bd in the wild (Murray et al. 2011; Murray and Skerratt in press).

While the discovery of chytridiomycosis has sparked renewed appreciation for the role that diseases can play in threatening wildlife populations and species, it is not the only disease currently affecting amphibians, nor is it likely to be the last. Ranavirus, for example, has been observed to induce mass mortality events in frog and salamander populations in the UK and North America. In response to these global threats, the World Organisation for Animal Health (OIE) has listed both chytridiomycosis and ranavirus as "notifiable" diseases to help control their spread. Similarly, numerous conferences and reports have been assembled to produce standards in managing diseases in wild and captive amphibian populations. Together, these measures highlight the importance of developing **agreed hygiene protocols for the control of diseases in Australian frogs**. This document fulfils this role.

## 4. Key disease issues in amphibian populations

Here we review the most significant diseases of amphibians, including some that have zoonotic potential and some that have not been detected in Australia. There are many described diseases of amphibians but only a few are known to be an important threat to wild amphibians or other taxa including humans. Some become an issue in captive amphibian populations where management is inadequate. As research on this topic is limited, there are also likely to be many unknown diseases of amphibians which may pose a risk. Disinfection methods have not been validated for all pathogens. Any risk management strategy to minimise the impact of diseases of amphibians should take into account this uncertainty. For detailed reviews see Hemingway et al. (2009) and Berger et al (2009) for diseases in wild populations and Wright and Whitaker (2001) that also includes diseases in captivity.

## 4.1. Fungi

### 4.1.1. *Batrachochytrium dendrobatidis*

*Batrachochytrium dendrobatidis* (Bd) is a fungal pathogen capable of driving amphibian species to perilously low numbers or extinction. In Australia, the oldest record of Bd is from a museum frog specimen collected in south-east Queensland near Brisbane in 1978 (Department of the Environment and Heritage 2006a), which coincides with sudden frog declines in a number of species and two species extinctions in the region (Berger et al. 1998; Hines et al. 1999). Subsequent amphibian declines in central coastal Queensland (1985-86) and the Wet Tropics (1990-95) suggest that *B. dendrobatidis* spread north to its current northern limit at Big Tableland near Cooktown (Laurance et al. 1996; Berger et al. 1999; Skerratt et al. 2010). In southern Australia, the spread of *B. dendrobatidis* is poorly documented but its distribution extends down the entire east coast to Tasmania (first detected in 2004) (Obendorf and Dalton 2006; Pauza and Driessen 2008). Two separate foci occur in other states, one in southwest Western Australia, where the earliest record dates to 1985, and another around Adelaide in South Australia (earliest record 1995) (Murray et al. 2010a). The Northern Territory is currently considered amphibian chytrid free (Skerratt et al. 2008; Skerratt et al. 2010; Murray et al. 2011).

In the majority of infected animals for most of the time, clinical signs of chytridiomycosis are absent. The period of showing signs is typically short and mostly limited to those amphibians that die. Central nervous system signs predominate, including behavioural change, slow and uncoordinated movement, abnormal sitting posture, tetanic spasms, loss of righting reflex and paralysis. Skin changes associated with chytridiomycosis are typically microscopic and not detectable at the clinical level with any degree of confidence, although abnormal skin shedding occurs (skin shed more frequently and in smaller amounts) and erythema (tissue reddening) of ventral surfaces and digits may be seen. For what to do if you encounter a sick or dead amphibian in Australia, see section 6.7. below. For a detailed factsheet about chytridiomycosis, see the Australian Wildlife Health Network website (http://www.wildlifehealth.org.au/AWHN/FactSheets/Fact_All.aspx).

### 4.1.2. *Mucor amphiborium*

This fungus is an important cause of morbidity and mortality in platypus in Tasmania and amphibians are a potential reservoir host (Gust et al. 2009). Amphibian mucormycosis is a systemic disease caused by the fungus, *Mucor amphibiorum*. Severely infected amphibians have fungi disseminated through their internal organs and skin. The fungi incite formation of granulomas that consist of inflammatory cells and fibrous tissue. At postmortem, the liver contains small pale nodules up to about 5 mm in diameter and usually in massive numbers. These nodules can also be seen in other organs such as the kidney, lung, mesentery, urinary bladder, subcutaneous sinuses and skin. The microscopic fungi are found inside these nodules. *M. amphibiorum* is a primary pathogen and can infect normal amphibians, but in the wild it appears to cause only sporadic infections. Possibly the usual inoculating dose in the wild is not high enough to cause epidemic disease. In captivity it can cause fatal outbreaks in collections. For more information on mucormycosis, see http://www.jcu.edu.au/school/phtm/PHTM/frogs/mucor/mucoramphibiorum.htm.

### 4.1.3. Oomycetes

Water moulds (family Saprolegniaceae, phylum Oomycota) are ubiquitous in surface water. High levels of infection with *Saprolegnia ferax* caused mortality of Western toad (*Bufo boreas*) egg masses in northwestern United States and were sufficient to affect local populations (Kiesecker et al. 2001). Epidemics may be associated with fish stocking or environmental cofactors.

## 4.2. Viruses

There are a number of viruses that are known to cause disease and mortality in amphibians, including ranaviruses, frog erythrocytic virus, Lucké tumor herpesvirus, herpes-like virus of skin, calicivirus and leucocyte viruses (Hemingway et al. 2009). In Europe and America the most important of these for their ability to cause mass mortalities and potentially population declines are the ranaviruses (Hyatt et al. 2000). Ranaviruses have been identified in a range of ectothermic vertebrates, including fish, amphibians (frogs, toads, salamanders) and reptiles (lizards, turtles, snakes). Some species can infect a broad host range across all these taxa.

Ranaviral disease is an emerging infectious disease overseas as it is being detected over an increasing geographic range and in more species (Hemingway et al. 2009). While ranaviral disease in wild amphibians has not been frequently observed in Australia, antibodies to ranaviruses have been detected widely (NSW, Qld, NT) in cane toads (*Bufo marinus*) (Zupanovic et al. 1998). Bohle iridoviris (BIV) was first found causing death in wild caught metamorphs of *Limnodynastes ornatus* and has since been detected in wild, moribund adult *Litoria caerulea* from Townsville and captive juvenile *Pseudophryne coriacea* from Sydney (Speare et al. 2001; Cullen and Owens 2002). Laboratory studies in Australia have also shown that cane toads (*Bufo marinus*) and a range of native frogs are susceptible to BIV (Speare et al. 2001). Tadpoles appear the most susceptible, while juvenile frogs were more susceptible than adults. Data on the geographical origin and time of emergence or introduction of ranaviruses in Australia is not known. Ranaviruses not currently found in Australia can cause disease in native Australian amphibians in experimental challenges; for example, Venezuelan Guatopo virus was able to kill *Litoria caerulea* in experimental trials (http://www.jcu.edu.au/school/phtm/PHTM/frogs/otherdiseases-viruses.htm). We need to prevent the introduction of pathogenic ranaviruses into Australia.

Clinical signs of acute ranaviral disease may be seen in tadpoles, metamorphs, juveniles and adults. In general, amphibians infected with ranavirus may show decreased activity, ascites (accumulation of fluid in the peritoneal cavity), anasarca (accumulation of serous fluid in various tissues and cavities of the body), skin ulceration, focal and systemic haemorrhages and death. For what to do if you encounter a sick or dead amphibian in Australia, see section 6.7. below. For a detailed factsheet about ranaviral disease, see the Australian Wildlife Health Network website (http://www.wildlifehealth.org.au/AWHN/FactSheets/Fact_All.aspx).

## 4.3. Bacteria

The range of bacteria reported as causing disease in amphibians is small. Bacterial septicaemia can cause significant disease in captivity. Infection with Aeromonas spp., non-haemolytic group B Streptococcus, Flavobacteria and chlamydia have caused outbreaks in captive amphibians and Mycobacteria can cause chronic problems. Another group of bacteria can be carried by amphibians with minimal effect and are potentially capable of causing

infections in humans (zoonotic diseases). Salmonella and Leptospira are in this category and are a potential risk to humans, livestock and domestic pets, see below.

### 4.4. Myxozoa

Myxosporean parasites (*Myxidium* spp.) in the brain and liver of declining Australian frogs, the Green and Golden Bell frog (*Litoria aurea*) and the Southern Bell frog (*Litoria raniformis*), have recently been reported to be associated with disease and may have a significant impact on wild frogs (Hartigan et al. 2011).

### 4.5. Mesomycetozoa

*Ichthyophonus sp.* occurs the USA where it is often an incidental finding in tadpoles, frogs and salamanders but may cause morbidity and mortality. It infects muscles and adult frogs with massive infections become lethargic and emaciated. Massive acute lethal infections with numerous mortalities occur infrequently in ranid larvae (D. Green, unpubl., Mikaelian et al. 2000)

### 4.6. Alveolates

A *Perkinsus*-like organism is a major cause of mortality events in tadpoles in the US. Occurs predominantly in tadpoles of *Rana* spp. and may cause mortality rates of 80-99% in a pond over the course of 2-6 weeks (Davis et al. 2007). Weakly swimming, bloated and floating tadpoles are found.

### 4.7. Zoonotic Diseases

Guidelines for preventing human exposure to amphibian disease are available at the Centre for Disease Control website- http://www.cdc.gov/healthypets/animals/reptiles.htm

#### 4.7.1. *Salmonella*

Amphibians may carry pathogenic *Salmonella* species, but rarely show signs of disease (Anver and Pond 1984). Prevalence of salmonellas isolated in clinically normal amphibians is generally greater than 10% and bacterial levels can be high (Sharma et al. 1974). In Australia, *Salmonella* were isolated from 12.7% (19/150) of *B. marinus* collected from the wild and 9 serotypes were identified. All nine had previously been isolated in Australia from humans and livestock (O'Shea et al. 1990). An outbreak of gastroenteritis in humans near Rockhampton possibly originated from green tree frogs (*Litoria caerulea*) contaminating drinking water in rainwater tanks (Taylor et al. 2000). Some strains of salmonellae are cosmopolitan while others are not found in Australia, but could be imported.

#### 4.7.2. *Leptospira*

*Leptospira* are spirochaetal bacteria that usually invade the kidney of vertebrates and are excreted in the urine. Humans and domestic animals are susceptible to various strains of *Leptospira* usually from the species *Leptospira interrogans*. Serious acute and chronic disease occasionally with death can result. Little is known about the occurrence of *Leptospira* in amphibians, and on their significance as reservoir hosts for leptospirosis in humans. No studies appear to have been done on leptospires in amphibians in Australia. However in

Barbados, toads (*Bufo marinus*) and frogs (*Eleutherodactylus johnstonei*) were found to be reservoirs for serovars of *Leptospira* pathogenic to humans (Gravekamp 1991).

### 4.7.3. *Spirometra erinacei*

The adult stage of the tape worm *Spirometra erinacei* inhabits the small intestine of carnivores such as the cat, dog, fox and dingo. The first larval stage occurs in copepods and the second larval stage (spargana) are long, flat white worms that can infect amphibians and other vertebrates in muscles and under the skin. Sparganosis occurs in around 5% of Australian frogs and heavy burdens are associated with severe disease (Berger et al. 2009). Sparganosis is a public health problem in Asia, usually occurring as subcutaneous or intramuscular infections. Humans become infected by drinking water with infected copepods, eating undercooked frogs, and the worms can also migrate from frog flesh into skin wounds

## 5. National and border biosecurity

Unregulated trade in animals, as well as unintentional shipment, is suspected to have been a major contributor to the spread of emerging infectious diseases such as chytridiomycosis (Skerratt et al. 2007). There are numerous bodies and regulatory levels that attempt to provide guidance about how to minimise the risk of pathogen spread and transmission in amphibians.

### 5.1. World Organisation for Animal Health (OIE)

The World Organisation for Animal Health (OIE) lists key diseases as "notifiable" to promote the reporting and management of diseases among member countries. Preventing the spread of amphibian diseases across international borders is important, and both chytridiomycosis (Article 8.1.1) and ranavirus (Article 8.2.1:) are now listed as notifiable diseases in the OIE Aquatic Animal Health Code (http://web.oie.int/eng/normes/fcode/). To access these codes, follow these links:

- **Chytridiomycosis**: http://web.oie.int/eng/normes/fcode/en_chapitre_1.8.1.pdf
- **Ranavirus**: http://web.oie.int/eng/normes/fcode/en_chapitre_1.8.2.pdf

The codes outline recommendations for the "**Importation or transit of *aquatic animals and aquatic animal products* for any purpose from a country, zone or compartment"**:

- **Provided commodities are treated in a manner that inactivates the disease agent (Bd or ranaviruses),** Competent Authorities should not require any disease conditions when authorising the above activities, regardless of the disease status of the exporting country
- However, in cases where it could otherwise reasonably be expected that commodities pose a risk of Bd or ranavirus transmission, a risk assessment should be carried out in accordance with the recommendations in the Aquatic Code. The exporting country would then be notified of the outcome of the risk assessment before trade commences.

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Where commodities do not meet this condition and/or a reasonable risk remains, there are additional requirements that depend on the disease status of the country, zone or compartment.

Freedom from disease:

Importation of live aquatic animals from a country, zone or compartment declared free from disease (Bd or ranavirus) requires an **international aquatic animal health certificate** issued by the Competent Authority confirming disease free status.

- A country may make a **self declaration of freedom from disease** (Bd or ranaviruses) if one of the following conditions is met:

    1. It has no amphibians or other susceptible species AND basic biosecurity conditions have been continuously met for a period of 2 years
    2. There has been no observed occurrence of the disease for at least the past 10 years despite conditions that are conducive to its clinical expression AND basic biosecurity conditions have been continuously met for a period of 10 years
    3. Targeted surveillance has been in place for at least the past 2 years without detection of disease (Bd or ranaviruses) AND basic biosecurity conditions have been continuously met for a period of 2 years
    4. For a country that previously made a self declaration of freedom from disease, it may regain that status after detection of the disease if the affected area was declared an infected zone and a protection zone was established AND infected populations have been destroyed or removed from the infected zone by means that minimise the risk of further spread of the disease AND the appropriate disinfection procedures have been completed AND if the conditions of 3.) above are met.

- A zone or compartment may also be declared free from disease by the Competent Authority if it meets similar conditions to the above. Where a zone or compartment extends over more than one country, declarations must be made by all the Competent Authorities involved.

- A disease free status can be maintained if basic biosecurity conditions are continuously maintained. Targeted surveillance may be discontinued provided conditions that are conducive to clinical expression of disease exist. However, in infected countries and in all other cases where conditions are not conducive to clinical expression of disease, zones or compartments can only maintain a disease free status if targeted surveillance is maintained.

Unknown or known infected country, zone or compartment:

For the importation of live aquatic animals and aquatic animal products for any purpose (e.g., aquaculture, processing for human consumption, use in animal feed, agricultural, laboratory, zoo, pet trade, industrial or pharmaceutical use):

In general, the Competent Authority of the importing country should

- require an **international aquatic animal health certificate** stating the commodities have been appropriately treated to inactivate disease agents
- OR undertake a risk assessment and apply appropriate risk mitigation measures

The risk assessment and risk mitigation measures will vary with purpose of the importation or transit of commodities. Please see the Aquatic Code at the links provided above for more details.

### 5.2. AUSVETPLAN and AQUAVETPLAN

In Australia, management of animal disease emergencies normally defaults to protocols outlined in the Australian Veterinary Emergency Plan (AUSVETPLAN - http://www.animalhealthaustralia.com.au/programs/eadp/ausvetplan/ausvetplan_home.cfm) or the Australian Aquatic Veterinary Emergency Plan (AQUAVETPLAN - http://www.daff.gov.au/animal-plant-health/aquatic/aquavetplan). However, few of the diseases for which specific plans have been developed concern diseases of free-ranging wildlife. No amphibian diseases are currently included in AUSVETPLAN or AQUAVETPLAN.

### 5.3. Key Threatening Process and Threat Abatement Plan (TAP)

Chytridiomycosis was listed as a Key Threatening Process in Australia in 2002. A Threat Abatement Plan (TAP) for infection of amphibians with chytrid fungus resulting in chytridiomycosis was subsequently prepared by representatives of the Commonwealth Government. These documents can be accessed here:

- **Key Threatening Process:** http://www.environment.gov.au/biodiversity/threatened/ktp/frog-fungus.html
- **TAP:** http://www.environment.gov.au/biodiversity/threatened/publications/tap/chytrid.html
- **TAP Background document:** http://www.environment.gov.au/biodiversity/threatened/publications/tap/pubs/chytrid-background.pdf

Recommendation 1.1.3 of the TAP proposes that a risk-based approach be used for chytridiomycosis using AUSVETPLAN as a model (Department of the Environment and Heritage 2006b). However, this has not progressed. Nation-wide mapping protocols and disease risk models have been developed as suggested in the TAP and should serve as the basis for cost-sharing arrangements between states and for setting research and management priorities (Skerratt et al. 2008; Murray et al. 2010a; Murray et al. 2010b; Skerratt et al. 2010; Murray et al. 2011). Implementing this step remains a priority.

### 5.4. Biosecurity Australia

Risk analysis performed by Biosecurity Australia in **"Quarantine requirements for the importation of amphibians or their eggs into zoological facilities"** and **"Quarantine requirements for the importation of amphibians or their eggs for laboratory purposes"** (Animal Biosecurity Policy Memorandum 2003/26) does not list chytridiomycosis as a risk since it is endemic in Australia. However, this disregards the risk of importation into chytrid free areas or the introduction of novel strains. Although chytridiomycosis is not specifically mentioned, the general hygiene strategies recommended should still prevent the release of imported strains of *B. dendrobatidis* during the initial two years. After two years the amphibians can be released without testing for *B. dendrobatidis*. However, if being released into a chytrid free area, the same requirements imposed on Australian bred amphibians under the Threat Abatement Plan would apply.

Risk analysis performed by Biosecurity Australia in **"Quarantine requirements for the importation of amphibians or their eggs into zoological facilities"** and **"Quarantine requirements for the importation of amphibians or their eggs for laboratory purposes"** (Animal Biosecurity Policy Memorandum 2003/26) mentions ranaviruses:

- "The veterinary certificate must… certify that… for both live amphibians or amphibian eggs…, as far as can be determined, no case of ranavirus infection (including frog virus 3, Redwood Park virus, Regina ranavirus), or ranid herpesviruses has been diagnosed at the premises of origin during the 12 months prior to certification."

Importation of amphibians must meet the requirements of two Commonwealth departments, 1) Department of Agriculture, Fisheries and Forestry (DAFF) and 2) the DSEWPaC. The relevant documents can be accessed here:

- **DAFF:**
  Zoological facilities - http://www.jcu.edu.au/school/phtm/PHTM/frogs/aqis/2003-26a.pdf
  Laboratory purposes - http://www.jcu.edu.au/school/phtm/PHTM/frogs/aqis/2003-26b.pdf
- **DSEWPaC:** http://www.environment.gov.au/biodiversity/wildlife-trade/index.html. This site also has the requirements for export of amphibians from Australia.

## 6. Hygiene management

Hygiene management issues can be broadly classed into *in-situ* (field based) and *ex-situ* (facility based) categories. While general **isolation and disinfection** hygiene management principles apply to both, greater detail on '**Guidelines for captive breeding, raising and restocking programs for Australian frogs'** can be found here: http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11.

### 6.1. In-situ (site) hygiene management

Individuals studying frogs often travel and collect samples of frogs from multiple sites. Numerous hygiene guidelines for handling wild frogs exist, including Daszak et al. (2001), NSW NPWS (2008), NWHC (2001), Speare et al. (2004) and CCADC (2008). Most recently, Phillott et al. (2010) provide a detailed review and synthesis of hygiene considerations that aim to minimise the risk of exposure of amphibians to pathogens in field studies.

It is important to recognise that humans may aid in the:

- **transmission** (passing of disease from an infected to an uninfected individual), and
- **spread** (movement of disease geographically)

of diseases, within and among amphibian populations  For researchers working with amphibians or within areas where amphibians may occur, the risk of disease transmission within these habitats and the spread of disease among populations may be increased due to:

- **movement** of frogs or personnel between isolated areas of habitat or between captive husbandry and laboratory facilities and the field
- **handling** of amphibians

It is therefore essential that personnel working with amphibians or within amphibian habitats take care to minimise disease transmission and spread. In order to do this, it is important that frog workers recognise the boundaries between sites/populations.

This is especially important where **rare, geographically restricted or threatened amphibian species** are concerned and when the spread of diseases can have serious consequences for species survival.

Phillott et al. (2010) recommend that field researchers evaluate their activities to determine the relative risk of pathogen transmission and spread compared with background levels (i.e., the risk posed by other mechanisms of disease transmission or pathogen dispersal) and implement appropriate strategies to minimise this risk during field studies. For a **hygiene protocol checklist and suggested field kit** see section 7. The risk of transmission and spread should also be evaluated by researchers, animal ethics committees and government agencies issuing permits.

### 6.1.1. Defining a site

Defining the boundary of a site may not be straightforward. In some places, the boundary between sites will be obvious but in others it may not. Undertaking work at a number of sites or conducting routine monitoring at a series of sites within walking distance creates obvious difficulties with boundary definitions. It is likely that defining the boundary between sites will differ among localities.

In general:

- watershed and geographical barriers should be used to designate separate sites
- river/stream tributaries should be considered separate sites
- wetlands, ponds, lakes etc. separated by dry land should be considered separate sites
- upstream locations separated by considerable distance (e.g., 500 m) should be considered separate sites
- any obvious break, barrier or change in habitats should be treated as separate sites, particularly if there is no known interchange of frogs between sites

### 6.1.2. Determining the order of visitation of multiple field sites

When a field trip encompasses several field sites, or a number of locations are being visited in succession, the order of visitation should be determined according to the presence of known pathogens and diseases.

- **Areas known to be absent of disease should be visited first, followed by areas of unknown status, followed by known infected areas**

### 6.1.3. On-site hygiene

When travelling from site to site it is recommended that the following hygiene precautions be taken to minimise the possibility of transfer of disease from personnel, footwear, equipment and/or vehicles. A list of suitable disinfectants, their required concentrations and exposure times for various purposes is summarised by Phillott et al. (2010) and is reproduced in Table 1 below.

**Personnel**

- **Hands, arms, knees etc. should be cleaned to remove debris and washed** or wiped with a suitable disinfectant. It is preferable to do this before entering the vehicle or moving to another site.

**Footwear and clothing**

- **Footwear must be thoroughly cleaned and disinfected** at the commencement of fieldwork and between each sampling site. This can be achieved by initially scraping boots clear of mud and standing the soles in a disinfecting solution. The remainder of the boot should be rinsed or sprayed with a disinfecting solution. Clothing that has significant contact with frogs and the environment should also be subjected to changing or cleaning

Disinfecting solutions should be prevented from entering any water bodies. Several changes of footwear/clothing bagged between sites might be a practical alternative to on-site cleaning. In high value sites, dedicated equipment and clothing stored at the entry to the site may be desirable. (e.g., in a lockbox)

**Equipment**

- Equipment such as nets, balances, callipers, bags, scalpels, headlamps, torches, wetsuits and waders etc. that are used at one site must be **cleaned and disinfected** before re-use at another site
- Disposable items should be used where practical/possible

Non-disposable equipment should be used only once during a particular field exercise and disinfected later or disinfected at the site between uses using procedures outlined below in Table 1.

**Vehicles**

Transmission of disease from vehicles is generally unlikely to be a problem. However, if a vehicle is used to traverse a known frog site and could result in mud and water being transferred to other bodies of water or frog sites, then wheels and tyres should be cleaned and disinfected. This is particularly important where vehicles are used in areas not normally frequented by other vehicles. Disinfection should be carried out at a safe distance from water bodies to minimise the risk of chemical contamination.

### 6.1.4. Principles of cleaning and disinfection

Designing an effective disinfection protocol requires understanding of the properties of disinfectants and target pathogens, and practical consideration of the equipment or processes requiring disinfection. As well as understanding the efficacy of various disinfecting processes, it is important to consider the safety of any disinfection protocol to the environment and the animals on which they will be used. Key distinctions include:

- **Cleaning**: The physical removal of all visible organic and inorganic debris from items
- **Disinfection:** A physical (e.g., UV light) or chemical (e.g., bleach) process to reduce the numbers and/or viability of microorganisms (e.g., bacteria, fungi or viruses) on an object, surface or material
- **Sterilization:** A physical or chemical process that removes all microorganisms from an object, surface or material

Thorough cleaning and disinfection reduces most of the risk of transferring amphibian pathogens. Sterilization of objects is labour intensive and less practical for most routine applications.

**Cleaning** alone does not render an object free of pathogens. However, it is important to thoroughly clean objects prior to disinfection or sterilization.

- Thorough cleaning physically removes many or most pathogens that are trapped in organic debris
- Thorough cleaning makes successful disinfection more likely
- Cleaning allows disinfectants to directly contact the surfaces of an object
- Warm or hot water improves the ability to remove organic materials from objects
- Regular cleaning of all items used should be performed
- Use of detergents aid cleaning by loosening organic material from the surface of objects and help to break apart biofilms of microorganisms that can resist disinfection
- Thorough rinsing of detergents from objects is essential after cleaning

**Disinfection** of an item by application of an appropriate chemical agent after cleaning reduces pathogen numbers and viability and minimises potential for disease transmission. Things to consider include:

- **Efficacy of the disinfectant and the type of pathogens that must be eliminated.** For example, some microorganisms such as Mycobacterium spp. or Cryptosporidium spp. are very resistant to most common disinfectants
- **The potential for toxicity to amphibians that are exposed to the disinfectant.** Amphibians are very sensitive to some disinfectant residues and thorough rinsing of all disinfectants is required after use
- **Concerns about human exposure to disinfectants and about discharge of disinfectants into the environment**
- **Safety for use on different materials.** Some disinfectants may be corrosive to materials or tools used in amphibian facilities
- **Ease of use and disposal**
- **Cost**

Table 1. Disinfection strategies suitable for killing *Batrachochytrium dendrobatidis*, *Mucor amphibiorum* and ranaviruses in field studies. From Phillott et al. (2010) and Webb et al. (submitted).

| Application | Disinfectant | Strength | Time | Target pathogen |
|---|---|---|---|---|
| Surgical equipment and other instruments (e.g. scales, callipers) | Benzalkonium chloride | 1 mg ml–1 | 1 min | *B. dendrobatidis* |
| | Ethanol | 70% | 1 min | *B. dendrobatidis*<br>Ranaviruses |
| Collection equipment and containers | Sodium hypochlorite (bleach contains 4% sodium hypochlorite) | 1% | 1 min | *B. dendrobatidis* |
| | | 3% | 1 min | Ranaviruses |
| | Path X or quaternary ammonium compound 128 | 1 in 500 dilution | 0.5 min | *B. dendrobatidis* |
| | | 1 in 100 dilution | 10 min | *M. amphibiorum* |
| | Trigene | 1 in 5000 dilution | 1 min | *B. dendrobatidis* |
| | F10 | 1 in 1500 dilution | 1 min | *B. dendrobatidis* |
| | Virkon | 2 mg ml–1 | 1 min | *B. dendrobatidis* |
| | | 1% | 1 min | Ranaviruses |
| | Nolvasan | 0.75% | 1 min | Ranaviruses |
| | Potassium permanganate | 1% | 10 min | *B. dendrobatidis* |
| | Complete drying | | >3 h | *B. dendrobatidis* |
| | Heat 60°C | | 30 min | *B. dendrobatidis*<br>Ranaviruses |
| | Heat 37°C | | 8 h | *B. dendrobatidis* |
| | Sterilising UV light | | 1 min | Ranaviruses only |
| Footwear | Sodium hypochlorite (bleach contains 4% sodium hypochlorite) | 1% | 1 min | *B. dendrobatidis* |
| | | 3% | 1 min | Ranaviruses |
| | Path X or quaternary ammonium compound 128 | 1 in 500 dilution | 0.5 min | *B. dendrobatidis* |
| | | 1 in 100 dilution | 10 min | *M. amphibiorum* |
| | Trigene | 1 in 5000 dilution | 1 min | *B. dendrobatidis* |
| | F10 | 1 in 1500 dilution | 1 min | *B. dendrobatidis* |
| | Phytoclean (30% benzalkonium chloride) | 0.075% | 1 min | *B. dendrobatidis* |
| | | 5% | 1 min | *M. amphibiorum* |
| | Complete drying | | >3 h | *B. dendrobatidis* |
| Cloth (e.g. carry bags, clothes) | Hot wash 60°C or greater | | 30 min | *B. dendrobatidis*<br>Ranaviruses |

### 6.2. Handling of frogs in the field

The spread of pathogens may occur as a result of handling frogs. In addition to spreading disease among captured frogs, handling may stress animals making them more susceptible to infection from other sources or more likely to succumb to infection.

- **Capture, handling and housing of wild amphibians should be minimised or avoided where possible**
- Where handling is necessary, care must be taken to ensure individuals do not have their exposure to pathogens elevated over their background exposure levels.

Direct transfer of pathogens during capture and handling of successive adult amphibians can be reduced by using:

- **single-use gloves** (latex, nitrile or vinyl), and/or
- **single-use lightweight plastic bags**
- **adequate cleaning of hands and handling equipment**

Many researchers use disposable plastic bags to catch and/or restrain frogs followed by handling/processing with disposable gloves. As some tadpoles may suffer lethal effects when exposed to latex, nitrile or vinyl gloves (Cashins et al. 2008), researchers should only use gloves that have been proven or rendered safe (e.g., by rinsing with water) for the study species.

In situations **where gloves are not available or suitable:**

- hand washing with 70% ethanol (allowing hands to dry) between handling individual frogs is acceptable (note, repeated use on human skin is not recommended). Alcohol is toxic to frogs so hands must be washed thoroughly in water after treatment with alcohol
    - If 70% ethanol is not available or suitable, the minimum treatment is hand-washing in the water to which the amphibian is normally exposed.

In situations **where amphibians must be held temporarily:**

- Individuals should be housed in **single-use containers (e.g. plastic bags) or in containers disinfected** between each animal
- Adults should not be held in groups
- Tadpoles from the same water body may be housed for short periods in a common container, although overcrowding should be avoided

Longer holding times (>60 min) will require changes to water and the provision of appropriate food (>24 h). Tadpoles should always be treated with care to prevent damage on capture and with movement of water within holding containers. If animals must be removed from the field for greater periods and later returned, it should always be to the same site.

### 6.3. Housing frogs and tadpoles

- **Frogs and tadpoles should only be removed from a site when absolutely necessary.**

Detailed **'**Guidelines for captive breeding, raising and restocking programs for Australian frogs' can be found at: http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11. See also 'A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs**'** (Pessier and Mendelson 2010) at: http://www.cbsg.org/cbsg/workshopreports/26/amphibian_disease_manual.pdf#search=%22amphibian%22

When frogs or tadpoles are to be collected and held for a period of time, the following measures are recommended:

- Isolate animals obtained at different sites
- Aquaria set up to hold frogs should not share water, equipment or any filtration system. Splashes of water from adjacent enclosures or drops of water on nets may transfer pathogens between enclosures
- Ensure that tanks, aquaria and any associated equipment are disinfected prior to housing frogs or tadpoles
- Tanks and equipment should be cleaned, disinfected and dried after frogs/tadpoles are removed

### 6.4. Marking, invasive and surgical procedures

Strict hygiene standards must be maintained during amphibian marking procedures including implanting internal radio transmitters, passive integrated transponder (PIT) tags, visible implant alphanumeric (VIA) tags, visible implant elastomer (VIE) tags and toe tipping or clipping.

Due to the high permeability of amphibian skin, special disinfectants are required.
The **only suitable, commercially available preparation for disinfecting wounds** is:

- **Bactine®** spray (active ingredient 0.14% w/w benzalkonium chloride and 2.6% w/w lidocaine hydrochloride in a non-alcohol base)
- **Chlorhexidine** (0.75% diluted from 2% Nolvasan®) is also suitable for surgical disinfection
- Alcohol, phenol and iodine based disinfectants **should not be used** because they are potentially toxic and can destroy mucus and wax that prevent dehydration and microbial infection of amphibian skin. Contrary to the recommendations of previous hygiene protocols, Betadine® or other povidone-iodine products are not recommended for use as disinfectants for amphibians until species-specific toxicity has been determined (Phillott et al. 2010).

Toe tipping (removal of most distal phalange) or toe clipping (amputation of a greater proportion of the digit):

- should occur through the **interphalangeal joints**

- Scissors should be **sterilised in 70% ethanol** and dried before use on frogs in the field
- For studies in which diagnostic testing of disease is important, the diagnostic test step (e.g., swabbing for Bd) should be undertaken before any other processing step to minimise the potential for false-positives due to cross contamination

PIT, VIE and VIA tags should be inserted with a **sterile, single-use applicator**.

### 6.4.1. Sealing wounds

- A c**ryanoacrylate** compound such as Vetbond® (active ingredient n-butyl cryanoacrylate) as a tissue adhesive after toe tipping or clipping is recommended. Vetbond® can also be used to seal incisions made during subdermal injection of VIA, VIE and PIT tags
- A disinfectant such as **Bactine**® should be applied before the adhesive to avoid trapping microbes
- Less expensive industrial adhesives ('superglues') should not be used as a replacement for surgical tissue glues

However, this procedure may only be possible in larger amphibians. In smaller animals, it can be difficult to isolate toes for application and internal marking devices such as PIT tags may be unsuitable. Moisture can interfere with setting times and adhesion so care must be taken to ensure setting has occurred before release. Problems may be experienced in their application to stream- or pond-dwelling amphibians, but can be avoided by using a small piece of sterile absorbent dressing to draw surplus water from the wound before application of the adhesive (Phillott et al. 2010).

### 6.4.2. Equipment

- Equipment used in marking or surgery should be appropriately **disinfected**
- Disposable sterile instruments should be used where practical/possible
- Instruments should be disinfected or changed in between each frog
- All used **disinfecting solutions, gloves and other disposable items should be stored in a sharps or other waste container and disposed of or sterilised appropriately** at the completion of fieldwork
- Disinfecting solutions must not come into contact with frogs or be permitted to contaminate any water bodies

## 6.5. Return of captive animals to the wild

- In general, if wild frogs or tadpoles are housed for any period of time in a captive situation (e.g. laboratory, zoo or captive breeding facility), **they should not be returned to the wild**

Exceptions to this can occur if they have been kept in isolation, their captive history is free of undiagnosed morbidity or mortality and they have had rigorous pathogen screening before release. This is usually beyond the means of most studies.

Detailed **'**Guidelines for captive breeding, raising and restocking programs for Australian frogs' can be found at:

http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11. See also 'A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs' (Pessier and Mendelson 2010) at: http://www.cbsg.org/cbsg/workshopreports/26/amphibian_disease_manual.pdf#search=%22amphibian%22

### 6.6. Displaced frogs

- **Displaced frogs should be treated as if they are infected and should not be transported anywhere for release to the wild**

Displaced frogs are native frog species and introduced cane toads (*Bufo marinus*) that have been unintentionally transported from one place to another. This may typically occur with the transport of fresh produce and landscaping supplies. 'Banana Box' frog is the term used to describe several native frog species (usually *Litoria gracilenta, L. fallax, L. caerulea, L. rubella, L. infrafrenata* and *L. bicolor*) commonly transported in fruit and vegetable shipments and landscaping supplies. There is risk of spread of disease if these frogs are transferred from place to place.

When encountering a displaced frog:

- Contact a **licensed wildlife carer** organisation to collect the animal. The frog may then undergo a quarantine period along with an approved disinfection treatment
- Post-quarantine, and dependant on local state legislation and policies, the frog may be transferred to a **licensed frog keeper** once permission from the relevant regulatory body has been received. Licensed carer groups are to record and receipt frogs obtained and disposed of in this way.
- Frogs held by licensed frog keepers are **not to be released to the wild** except with relevant regulatory body approval

Displaced frogs may also be made available to recognised institutions for research projects, display purposes or offered to a museum as scientific specimens once approval has been provided by the relevant regulatory body.

- **Frogs encountered on roads, around dwellings and gardens or in swimming pools should not be considered as displaced frogs unless they are of a species not local to the area**

Local frogs encountered in these situations should be assisted off roads, away from dwellings, or out of swimming pools preferably to the nearest area of vegetation or suitable habitat.

#### 6.6.1. Cane toads

**Cane toads are known amphibian disease carriers and should not be knowingly transported or released to the wild.**

If a cane toad is discovered it should be humanely euthanized in accordance with the recommended Animal Welfare procedures. Care should be taken to avoid euthanasia of native species due to mistaken identity.

### 6.7. Sick and dead animals

Dead amphibians or live animals showing clinical signs of disease must be regarded as having a high infection risk to healthy animals and rigorous hygiene measures are required.

- **Sick and dead frogs should be collected and sent for disease diagnosis**

No effective and practical field treatment for chytridiomycosis has been demonstrated. Similarly, no treatment regimes for ranaviral infection of frogs have been described. The collection of sick and dead frogs for expert diagnosis may improve disease surveillance activities, which can help detect disease introduction and enable emergency responses. It is also useful to assess the risk of pathogen transmission to other individuals or spread to other populations. A procedure for the preparation and transport of a sick or dead frog is given below. Adherence to this procedure will ensure the animal is maintained in a suitable condition for pathological examination and assist determining the extent of the disease and the number of species affected. For more information about sick and dead amphibians, see http://www.jcu.edu.au/school/phtm/PHTM/frogs/pmfrog.htm.

Collection:

- Do not use bare hands to handle sick or dead frogs
- Disposable gloves should be worn when handling sick or dead frogs
- New gloves and a clean plastic bag should be used for each frog specimen to prevent cross-contamination
- If the frog is dead, keep the specimen cool and preserve as soon as possible to avoid decomposition

Preserving specimens:

- Specimens can be **preserved/fixed in 70% ethanol or 10% buffered formalin**
- Cut open the belly and place the frog in about 10 times its own volume of preservative
- Where no preservative is available, **specimens can also be frozen**. If numerous frogs are collected, some should be preserved and some should be frozen. Portions of a dead frog can also be sent for analysis (e.g., a preserved foot, leg or a portion of abdominal skin)

Transportation:

- **If the frog is alive and likely to survive transportation**, place the frog into either a moistened cloth bag with some damp leaf litter or into a plastic bag with damp leaf litter and partially inflated before sealing
- Remember to **keep all frogs separated** during transportation
- **If the frog is alive but unlikely to survive transportation** (death appears imminent), euthanize the frog and place the specimen in a freezer or preservative. Once frozen/preserved the specimen is ready for shipment
- **All containers should be labelled** showing at least the species (if known), date and collection location
- Preserved samples can be sent in jars or wrapped in wet cloth, sealed in bags and placed inside a padded box
- Send frozen samples in an esky with dry ice

- Place live or frozen specimens into a small Styrofoam esky. Seal esky with packaging tape before sending
- Send the package by courier and declare any hazardous or flammable contents (e.g., 70% ethanol)

## 7. Hygiene protocol checklist and field kit

The following checklist and field kit are designed to assist with minimising the risk of transferring pathogens between frogs and sites in field studies (follows NSW 2008)

**Have you considered the following questions before handling frogs in the field:**

- Has your proposed field trip been sufficiently well planned to consider hygiene issues?
- Have you considered the boundaries between sites (particularly where endangered species or populations at risk are known to occur)?
- Have footwear disinfection procedures been considered and a strategy adopted?
- Have you planned the equipment you will be using and developed a disinfection strategy?
- Are you are planning to visit sites where vehicle disinfection will be needed? If so, do you have a plan to deal with vehicle disinfection?
- Have handling procedures been planned to minimise the risk of frog to frog pathogen transmission?
- Do you have a planned disinfection procedure to deal with equipment, apparel and direct contact with frogs?

**If you answered NO to any of these questions please re-read the relevant section of the *Hygiene Protocols for the Control of Disease in Australian Frogs* and apply a suitable strategy.**

### Field hygiene kit

When planning to survey frogs in the field a portable field hygiene kit should be assembled to assist with implementing the hygiene protocols. Recommended contents of a field hygiene kit would include:

- Plastic box to store field equipment
- Small Styrofoam esky
- Disposable gloves
- Disinfectant spray bottle (atomiser spray) and/or wash bottle for disinfectants
- Disinfecting solutions
- Scraper or scrubbing brush for cleaning mud off footwear, vehicles etc.
- Bucket for mixing disinfecting solutions and soaking
- Plastic bags, large and small for hygienic temporary animal handling/holding
- Sharps or other container for safe waste disposal
- Materials for dealing with sick and dead frogs (see section 6.7.)

Detailed 'Guidelines for captive breeding, raising and restocking programs for Australian frogs' can be found at: http://www.environment.gov.au/biodiversity/invasive/projects/index.html#threat-10-11. See also 'A Manual for Control of Infectious Diseases in Amphibian Survival Assurance Colonies and Reintroduction Programs' (Pessier and Mendelson 2010) at: http://www.cbsg.org/cbsg/workshopreports/26/amphibian_disease_manual.pdf#search=%22amphibian%22

## 8. Important Australian contacts

### 8.1. Sick and dead frogs

To arrange receipt and analyse sick and dead frogs, make contact with experts at any of the organisations below prior to dispatching package:

Australian Registry of Wildlife Health
Taronga Conservation Society,
Australia
PO Box 20
MOSMAN NSW 2088
Phone: 02 9978 4749

School of Public Health, Tropical Medicine and Rehabilitation Sciences
James Cook University
Douglas Campus
TOWNSVILLE QLD 4811
Phone: 07 4796 1735

School of Biological Sciences
University of Newcastle
CALLAGHAN NSW 2308
Phone: 02 4921 6014

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