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A survey of *Edwardsiella ictaluri* in wild catfish populations in Australia

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Abbreviations

ESC: enteric septicaemia of catfish

Executive Summary

This report contains the findings of the first survey of the exotic bacterium *Edwardsiella ictaluri* in wild freshwater fish populations in Australia. *Edwardsiella ictaluri* causes enteric septicaemia of catfish (ESC), which is a serious disease of farmed channel catfish in the USA. The bacterium has previously been detected in imported ornamental fish and in native catfish held in Australian aquarium facilities, but wild fish populations in Australia are considered free of the disease. The Australian Government Department of Agriculture, through the Fisheries Research and Development Corporation, funded an active surveillance program to provide further evidence for this claim of disease freedom.

Background

It is never possible to *prove* that a population is free from a disease-causing pathogen. It is possible, however, to estimate the probability that the population is free from the pathogen at a given level of infection. This requires an appropriate sampling strategy to survey high-risk individuals and a diagnostic test of sufficient sensitivity to detect the pathogen if it is present. This study targeted wild catfish species (because catfish are known to be susceptible to *E. ictaluri*) in northern Australia (because acute ESC disease occurs at higher temperatures) and concentrated our sampling around population centres (because the most likely source of *E. ictaluri* is from the release of infected ornamental fishes).

Aims

1) Design a targeted survey for *E. ictaluri* in wild catfish in rivers in northern Australia to establish disease freedom with 95% confidence at a prevalence of less than 5%.

2) Conduct an active survey of wild catfish populations in river systems in northern Australia for the presence of *E. ictaluri* by appropriate laboratory tests.

Methodology

We developed a risk-based sampling model and used this model to test different survey designs. Our final design, based on the model, involved a mean sample size of 18 fish from each of 15 sites, providing a probability of 95% that wild populations of catfish in northern Australia are free of *E. ictaluri* at an overall prevalence 1%, given negative survey results.

Catfish were sampled from these sites, with the assistance of a large network of collaborating freshwater fish scientists. Tissue samples were cultured for evidence of *E. ictaluri* and, if potentially positive cultures were found, DNA sequencing was used to confirm identity.

Results

Edwardsiella ictaluri was detected in eight *Tandanus tropicanus* catfish sampled at one site in the Tully River in northern Queensland. No infected catfish were found at any other site in Queensland, the Northern Territory or Western Australia, although it is possible that the bacterium is present at these sites at low prevalence.

Implications

Since *Edwardsiella ictaluri* was found at one of the sites surveyed, Australia cannot be considered to be free from *E. ictaluri*. Because the bacterium can survive in the bottom sediments of rivers, eradication at that site is probably not feasible. If *E. ictaluri* is present only in the Tully River, it may be possible to minimise the potential for spread through appropriate management actions. While the survey did not find any evidence of infection at other sites, it is possible that it is present at low prevalence.

Recommendations

1) As an immediate response, we recommend that actions be taken to minimise the risk of the spread of *E. ictaluri* from the Tully River to other localities in northern Australia.

2) Recommendation (1) makes the provisional assumption that infection is confined to the Tully River. This has not yet been established. To determine whether the bacterium is isolated to the one site at which it was detected or is more widespread will require a sampling regime that would detect low prevalence of infection, but a cost-benefit assessment would need to be undertaken prior to sampling.

3) If it is determined from further sampling that *E. ictaluri* is localised to the Tully River, then additional management activities may be required and these should be guided by a risk assessment. There are some key information gaps that need to be filled to inform this risk assessment process. Of particular importance is the susceptibility and tolerance of Australian native fish species to infection by *E. ictaluri*.

Keywords

Disease freedom; *Edwardsiela ictaluri*; ESC; catfish; *Tandanus tropicanus*

Introduction

Invasive species and co-invaders

Invasive species are alien (non-native) organisms that have been introduced into an area outside of their natural range, established self-sustaining populations and spread beyond their initial point of introduction, with deleterious impacts on the environment, the economy or human health (Kolar and Lodge 2001). Human population growth, increasing transport capacity and economic globalisation have accelerated the rate of introductions of alien species throughout the world (Sakai *et al.* 2001). Invasive species are now recognised as a major cause of biodiversity loss and associated changes in ecosystem function (Simberloff 2011).

Invasive species may affect native species directly, through competition or predation, or indirectly, by altering habitat or changing disease dynamics. If alien hosts introduce new parasites (using the term to include both microparasites, such as viruses and bacteria, and macroparasites, such as protozoa, helminths and arthropods), then these may be transmitted to native hosts, leading to the emergence of new disease in the natives (spillover or pathogen pollution; Daszak *et al.* 2000). To threaten native hosts in a new locality, alien parasites must overcome the same barriers to introduction, establishment and spread as free-living aliens and, in addition, they must be able to switch from alien to native hosts (Figure 1).

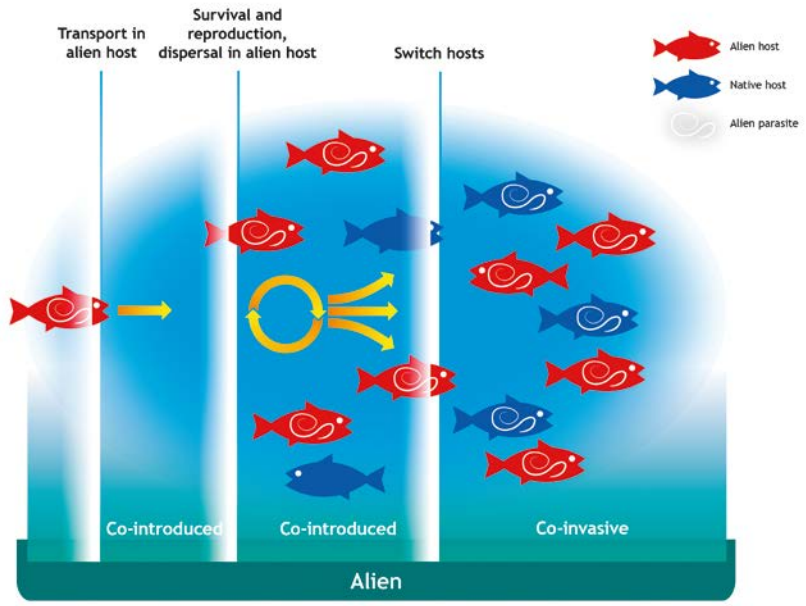

Figure 1. Schematic diagram of processes involved in species co-invasion. The light blue oval shape represents a new area, outside the natural range of the alien host species, shown in red. The alien host species contains an alien parasite species. Arrows indicate movement of alien host species through the phases of introduction, establishment and invasion of the habitat of the native host species, shown in blue. Vertical bars represent barriers to be overcome in each phase. The term co-introduced is used for those parasites which have entered a new area outside of their native range with an alien host, and co-invader for those parasites which have been co-introduced and then switched to native hosts. The alien parasite goes through the processes of introduction, establishment and spread with its original host and then switches to a native host species to become a co-invader. Adapted from Lymbery *et al.* (2014).

In a review of 98 cases of co-introductions throughout the world, Lymbery *et al.* (2014) found that fishes were by far the most common alien hosts in published studies, making up 55% of the total, with 81% of fish hosts being either freshwater or diadromous. This may reflect a taxonomic bias in studies, but is also likely due to the propensity for freshwater ecosystems to be particularly affected by invasive fishes (Johnson and Paull 2011).

Invasive freshwater fishes and co-invaders in Australia

Alien fish species were first introduced into Australia by European settlers in the late 18th and early 19th century and there are now 35 invasive species with established wild breeding populations in Australia, of which 22 are imported ornamental species (Lintermanns 2004). An estimated 10 to 16 million live ornamental fishes are imported into Australia annually, making this the major pathway for alien fish introduction (Department of Agriculture, Fisheries and Forestry 2006).

Invasive ornamental fishes have also introduced a number of co-invading parasites to Australia, including: viruses, such as Gourami iridovirus (Go and Whittington 2006) and Cyprinid herpesvirus 2 (Stephens *et al.* 2004); bacteria, such as *Aeromonas salmonicida* (Humphrey and Ashburner 1993); and eukaryotes, such as *Ichthyophthirius multifiliis* (Ashburner 1976), *Trichodina heterodentata, T. mutabilis, T. reticulata* and *T. acuta* (Dove and O'Donoghue 2005), *Bothriocephalus acheilognathi* (Dove and Fletcher 2000) and *Lernaea cyprinacea* (Dove 2000; Marina *et al.* 2008). Quarantine policies in Australia are based on risk analysis guidelines established under the World Trade Organisation's Sanitary and Phytosanitary (SPS) Agreement. Risk analysis procedures assess the needs for measures based on current knowledge and therefore cannot account for unforeseen effects of introduction of exotic pathogens, and may not fully address the high risks associated with the large numbers of ornamental fish species traded internationally, the large number of exotic parasites recorded in these species, poorly defined epidemiological and pathogenic data for the parasites, lack of post-border controls, the propensity for introduced alien fishes to establish breeding populations in the wild, and the ability of many co-introduced parasites to infect native fishes (Whittington and Chong 2007). These factors make it difficult both to identify hazards, and to determine the likelihood and consequences of establishment.

Edwardsiella ictaluri

Edwardsiella ictaluri is the causative agent of enteric septicaemia of channel catfish *Ictalurus punctatus* (ESC; Hawke *et al.* 1981). Acute ESC has been observed most frequently in cultured *I. punctatus* at water temperatures between 20 and 30ºC (Francis-Floyd *et al.* 1987; Shotts and Plumb 2003). It is characterised externally by ulcers and pin point haemorrhages, with diffuse internal septicaemia and tissue necrosis (Shotts *et al.* 1986; Hawke *et al.* 1998; Evance *et al.* 2011). Mortality rates typically range from 10-50% (Hawke and Khoo 2004). Fish which recover from acute infection develop a specific immune response and may carry the pathogen for extended periods (up to 200 days) (Klesius 1992; Mqolomba and Plumb 1992; Hawke *et al.* 1998). There is also a chronic form of disease, characterised by meningoencephalitis (Newton *et al.* 1989), and fish can be infected asymptomatically (Klesius 1992; Chen *et al.* 1994). There is no evidence for age or sex differences in disease susceptibility of naïve fish (Plumb and Hanson 2011; Peterson and Davis 2012).

Where *E. ictaluri* is present, prevalence rates are typically high. In a survey of channel catfish farms in the USA, 78.1% of all operations and 42.1% of all ponds experienced problems with ESC (Wagner *et al.* 2002). Klesius (1992) found that prevalence of *E. ictaluri* increased from 40% to 70% in a population of channel catfish following treatment and recovery from ESC. Studies of *E. ictaluri* in wild populations of fish are rare, but Hassan *et al.* (2012) found prevalence varying from 3% to 70% in a three year longitudinal study of subclinical infection of wild ayu (*Plecoglossus altivelus*) in Japan. Infection rates were higher in the cooler months of the year, when fish spawn.

Edwardsiella ictaluri has been isolated from seven families of catfish (Ictaluridae, Bagridae, Clariidae, Pangasiidae, Siluridae, Plotosidae and Ariidae) and from an increasing number of non-catfish species

throughout the world (Table 1). It appears to be a host generalist, although a few (non-catfish) species have been found to be resistant to experimental infections (Plumb and Sanchez 1983).

Table 1. Host species and geographic range of *Edwardsiella ictaluri*.

Host family	Host species	Geographic area	Reference
Ictaluridae	*Ictalurus punctatus*	USA[1]	Hawke (1979)
	I. furcatus	USA[5]	OIE (2009)
	I. catus	USA[5]	Shotts and Plumb (2003); OIE (2009)
	Ameiurus natalis	USA[5]	Shotts and Plumb (2003); OIE (2009)
	A. nebulosus	USA[5]	Shotts and Plumb (2003); OIE (2009)
	A. melas	USA[5]	Shotts and Plumb (2003); OIE (2009)
	Noturus gyrinus	USA[5]	OIE (2009)
Bagridae	*Pelteobagrus fulvidraco*	China[1]	Ye *et al.* (2009)
Clariidae	*Clarias batrachus*	Thailand[3]	Kasornchandra *et al.* (1987)
Pangasiidae	*Pangasius bocourti*	Thailand[1]	Dong *et al.* (2015)
	Pangasianodon hypophthalmus	Vietnam[1]	Crumlish *et al.* (2002)
		Sumatra[1]	Yuasa *et al.* (2003)
Plecoglossidae	*Plecoglossus altivelis*	Japan[3]	Sakai *et al.* (2008); Hassan *et al.* (2012)
Siluridae	*Silurus meridionalis*	China[1]	Geng *et al.* (2013)
Plotosidae	*Anodontiglanis dahli*	Australia[2]	Animal Health Australia (2012)
	Neosilurus ater	Australia[2]	Animal Health Australia (2012)
Ariidae	*Neoarius berneyi*	Australia[2]	Animal Health Australia (2012)
Sternopygidae	*Eigenmannia virescens*	USA[1]	Kent and Lyons (1982)
Cyprinidae	*Danio rerio*	USA[1,4]	Petrie-Hansen (2007); Hawke *et al.* (2013)
	Devario devario	USA[1]	Waltman *et al.* (1985)
	Pethia conchonius	Australia[2]	Humphrey *et al.* (1986)
Cichlidae	*Sarotherodon aureus*	USA[4]	Plumb and Sanchez (1983)
	Oreochromis niloticus	St. Kitts[1]	Soto *et al.* (2012)
Salmonidae	*Oncorhynchus tshawytscha*	USA[4]	Baxa *et al.* (1990)
	Oncorhynchus mykiss	Turkey[1]	Keskin *et al.* (2004)

[1]Cultured fish; [2]Quarantine facilities; [3]Wild populations; [4]Experimental infection; [5]Unknown origin

Edwardsiella ictaluri in Australia

The first report of *E. ictaluri* in Australia was in imported rosy barbs, *Puntius conchonius* (Humphrey *et al.* 1986). In 2011, *E. ictaluri* was detected in native Australian Berney's catfish, *Neoarius berneyi*, toothless catfish, *Anodontiglanis dahli*, and black catfish, *Neosilurus ater*, held in tanks in the same facility as imported ornamental fishes, suggesting that Australian catfish are susceptible to *E. ictaluri* infection (Animal Health Australia 2012). To date, *E. ictaluri* has not been reported in wild fishes in Australia, although no comprehensive survey has been undertaken.

Determining the absence of *E. ictaluri* in wild fish populations in Australia is important for two reasons. First, infection is often associated with high mortality rates and may therefore represent a threat to Australia's unique freshwater fish fauna. Infectious diseases are being increasingly recognised as important drivers of species declines and extinctions (Daszak *et al.* 2000; Harvall *et al.* 2002; Smith *et al.* 2006; Thompson *et al.* 2010). Second, if *E. ictaluri* is in Australia, this may have consequences for Australia's growing ornamental fish industry. This industry, inclusive of breeding facilities, wholesale traders, retail outlets and hobbyists was valued at $350 M in 2005 (Tilzey 2005), with up to 15 million fish imported and 700 thousand exported per year (O'Sullivan *et al.* 2008). To minimise disease spread, government authorities in Australia and overseas may require quarantine measures to be applied to imported products. The Sanitary and Phytosanitary agreement of the World Trade Organisation requires that quarantine measures should be based on a transparent scientific risk analysis, part of which requires science-based assessment of the risks of spread of diseases associated with the traded commodities. If Australia is free of *E. ictaluri*, then the risk of exporting the bacterium in Australian products is zero; however, if *E. ictaluri* is present in Australia, then Australian products may meet with additional quarantine restrictions. From an importation perspective, a demonstration of freedom from disease or, if the disease is present, the establishment of an official control program, may justify the imposition of additional quarantine measures on imported ornamental fishes.

Establishing freedom from disease

Two approaches have commonly been used to demonstrate freedom from disease; a structured, representative survey of the relevant population and qualitative assessment of multiple sources of evidence by a panel of experts (Martin *et al.* 2007). Both approaches have significant weaknesses. Structured surveys may be expensive, difficult to implement and ephemeral in their conclusions. Qualitative assessments may be heavily influenced by the assessors involved and therefore suffer from problems with transparency and repeatability. Martin *et al.* (2007) proposed a general methodology based on stochastic scenario tree modelling, which enables multiple sources of evidence to be used in developing a quantitative probability estimate of freedom from disease. For a single component of a surveillance system (e.g. a serological survey) the method partitions the reference population into groups within which all units have the same probability of being detected as infected; this allows the sensitivity of detection to be calculated for both representative (random) and targeted sampling schemes. For multiple components (e.g. schemes with different surveillance systems or both quantitative and qualitative assessments), the sensitivity of the combined components can be estimated by Bayesian techniques (Martin *et al.* 2007; Hood *et al.* 2009).

Objectives

1) Design a targeted survey for *E. ictaluri* in wild catfish in rivers in northern Australia to establish disease freedom with 95% confidence at a prevalence of less than 5%.

2) Conduct an active survey of wild catfish populations in river systems in northern Australia for the presence of *E. ictaluri* by appropriate laboratory tests.

Methodology

Survey design

We used the scenario tree method of Martin *et al*. (2007) to design a risk-based survey for *E. ictaluri* in northern Australian native catfish. This method allows the quantification of the sensitivity of a non-random sampling approach that stratifies the population by factors affecting the probabilities of infection and detection. A complete description of the approach is available in Appendix 1. Briefly, we developed a model of the survey process, defining all ways in which a positive outcome (isolation of *E. ictaluri*) can be obtained at user-defined design prevalences. The model, implemented in Microsoft Excel with the PopTools add-in, allowed us to calculate the survey sensitivity (*SSe*; the probability of detecting infection given that it is present). From this, it is possible to calculate the probability that the survey region is free from *E. ictaluri*, given negative survey results (*pFree*) from:

$$pFree = \frac{1 - PriorPInf}{1 - PriorPInf \times SSe}$$

where *PriorPInf* is the pre-survey probability of infection in the survey region, arbitrarily assigned a value of 0.5. A number of assumptions (see Appendix 1 for a full explanation) were made in developing the model.

1) All catfish species in northern Australia are equally susceptible to *E. ictaluri*.

2) There are no age- or sex-dependent differences in susceptibility.

3) The most likely source of infection is through the release of infected ornamental fishes, and for cities or towns in Australia with more than 5,000 people, there is a strong relationship between population size and number of aquarium shops (see Appendix 1). We therefore considered proximity to human population centres as the major risk factor for infection and sampling sites were chosen on that basis.

4) The majority of fishes harbouring infection will be recovered carriers, and our sampling techniques, which target actively swimming fish, have an equal chance of catching these fishes or uninfected fishes. Sampling is therefore assumed to be random after the population has been stratified by risk factors.

5) Diagnostic test sensitivity is 0.80, and specificity is 1.0.

Our model simulations included 31 of the approximately 55 major river systems throughout northern Australia (see Appendix 1). All rivers with population centres containing more than 5,000 people were included in the simulations, as well as a selection of rivers which did not have large population centres. These "low-risk" rivers were included to increase the geographic coverage of the survey. Model simulations identified a range of sampling options, all of which provided a probability of 95% that wild populations of catfish in northern Australia are free of *E. ictaluri* at a prevalence of 5% or less, given negative survey results. Our final design, chosen on the basis of cost-effectiveness from among these options , involved a mean sample size of 18 fish from each of 15 sites, and provided 95% confidence of disease freedom at an overall prevalence of 1% (among-river and within-river design prevalences of 10% each).

The final sampling design included ten "high risk" rivers (i.e. those with population centres of 5,000 people or more) and 5 "low risk" rivers. Sampling sites along the rivers were chosen on accessibility and presence of catfish populations (where such information was available). "High risk" rivers were sampled as close as possible to population centres, with different risk categories assigned for sampling within, downstream and upstream of town boundaries (see Appendix 1). Some adjustments in

sampling design were required during the survey, because of difficulty in accessing or capturing fishes from certain sites. Replacement sites were chosen to have similar characteristics as initial design sites, and these changes did not affect the probability of disease freedom in model simulations.

Sampling

Fishes were collected from 15 localities throughout northern Australia (Table 2, Figure 2). Most fishes were captured in fyke nets of 2 mm woven mesh, with width, length and depth varying depending on the characteristics of the site. In a small number of localities, fyke nets could not be set and fish were captured using seine nets and line fishing. At least 20 fishes were sampled from each locality except the Bloomfield River, where 19 fish were collected, the Ashburton River, where 18 fish were collected, and the Ross River, where only 16 fishes could be captured. Sampled fishes were transported to laboratory holding facilities and kept in well aerated aquaria until they were euthanized for examination. After each sampling session, all equipment was disinfected in chlorine solution, then washed in clean water and air dried before being used at another locality.

Fishes were euthanized using a prolonged anaesthetic bath of AquiS (isoeugenol). Weight, body length measurements and any external or internal gross abnormalities were recorded. Fish condition was estimated from the residuals of the regression of weight on body length. Fish tissues were then collected by standard necropsy procedures (http://www.agriculture.gov.au/biosecurity/risk-analysis/ira/current-animal/ornamental-finfish/ornamental-fish-testing-project-final-report/appendix-2). For bacterial isolation, a sample of spleen and kidney tissue was taken, followed by a sample of intestinal tissue. Pooled spleen and kidney tissue, and intestinal tissue were homogenised separately by crushing with a sterile inoculation loop within a sterile eppendorf tube, and then inoculated onto blood agar (BA; 3% horse blood agar, PathWest Laboratory Medicine WA) and *E. ictaluri* medium (EIM) plates, prepared according to Shotts and Waltman (1990). Inoculated plates were couriered immediately in sturdy insulated boxes under ambient temperatures to the Animal Health Laboratories, Department of Agriculture and Food Western Australia. Transit time from sample collection to arrival was typically 12 hours.

All fish dissections and inoculation procedures were performed indoors to minimise dust contamination; in a laboratory if one was available within one hour of the sampling site or in in temporary laboratory facilities erected in the field. To prevent cross-contamination, the necropsy table, dissecting board and all instruments were cleaned with ethanol between fish dissections. Once the abdominal cavity was opened, and between collection of different tissue samples, all dissecting instruments were again cleaned with ethanol and allowed to air dry. Inoculation loops were used only once.

Isolation and identification of *Edwardsiella ictaluri*

On arrival at the Animal Health Laboratories, inoculated plates were incubated overnight at 24^0C. Bacteria were identified using the MALDI-TOF (matrix assisted laser desorption ionisation time of flight mass spectrometer) Biotyper from Bruker Daltonics. Isolates identified to species level as being *E. ictaluri* were further confirmed using conventional biochemical tests according to Buller (2015) and molecular techniques. For molecular testing, DNA was extracted from a pure growth of bacterial cells using the PrepMan Ultra Reagent (Applied Biosystems) and tested using the primers designed according to Williams and Lawrence (2010). Identification was checked by performing 16S rRNA sequencing on one positive sample. Amplified product was purified using the QUIquick PCR purification kit (Qiagen) and sent to the Australian Genome Research Facility for sequencing. Positive samples (both culture and DNA) were sent to the Fish Diseases Laboratory at the Australian Animal Health Laboratory for confirmation using specific PCR and 16S rRNA sequencing.

Bacteria identified as being *Aeromonas* or *Vibrio* species by MALDI-TOF were confirmed to species level using conventional biochemical tests (Buller 2015). As stated on the MALDI-TOF database, species within the genera *Aeromonas* and *Vibrio* are so closely related that a very high probability score (≥2.3) is required for confidence to species level. All isolates identified as *Vibrio cholerae* were

checked by biochemical identification and serotyping for 01 and 0139 serotypes. These serotypes cause cholera in humans and are exotic to Australia. Only *Vibrio cholera* non-01 serotype was detected.

Table 2. Sample sites, locations and fish species captured from each site.

Site ID	River	Latitude (°S)	Longitude (°E)	Fish species collected (n)
BLR	Logan R	27.7609	153.0670	*Neoarius graeffei* (20)
BBR	Brisbane R	27.5447	152.7837	*N. graeffei* (20)
TCM	Mary R	26.3319	152.7020	*Tandanus tandanus* (18), *Neosilurus hyrtlii* (1), *N. graeffei* (1)
SPM	Mary R	26.0342	152.5106	*T. tandanus* (9), *N. graeffei* (11)
BYB	Burnett R	25.2304	152.0116	*N. graeffei* (20)
MPR	Pioneer R	21.1540	148.7266	*T. tandanus* (20)
TRR	Ross R	19.3232	146.7360	*Neosilurus ater* (15), *N. hyrtlii* (1)
CTU	Tully R	17.8818	145.8412	*Tandanus tropicanus (*20)
CBA	Barron R	17.2611	145.5378	*T. tandanus* (20)
CBI	Bloomfield R	15.9868	145.2882	*T. tropicanus* (19)
DRC	Rapid Creek	12.3955	130.8722	*N. hyrtlii* (30)
NTD	Daly R	13.6780	130.6439	*N. graeffei* (20), *Neoarius leptaspis* (2), *N. ater* (1)
KLK	Ord R	15.7932	128.7177	*N. graeffei* (14), *Neoarius midgleyi* (13)
KSC	Fitzroy R	17.9924	124.2023	*N. hyrtlii* (10), *N. graeffei* (7), *N. ater* (3)
PAR	Ashburton R	21.7777	114.9817	*N. graeffei* (18)

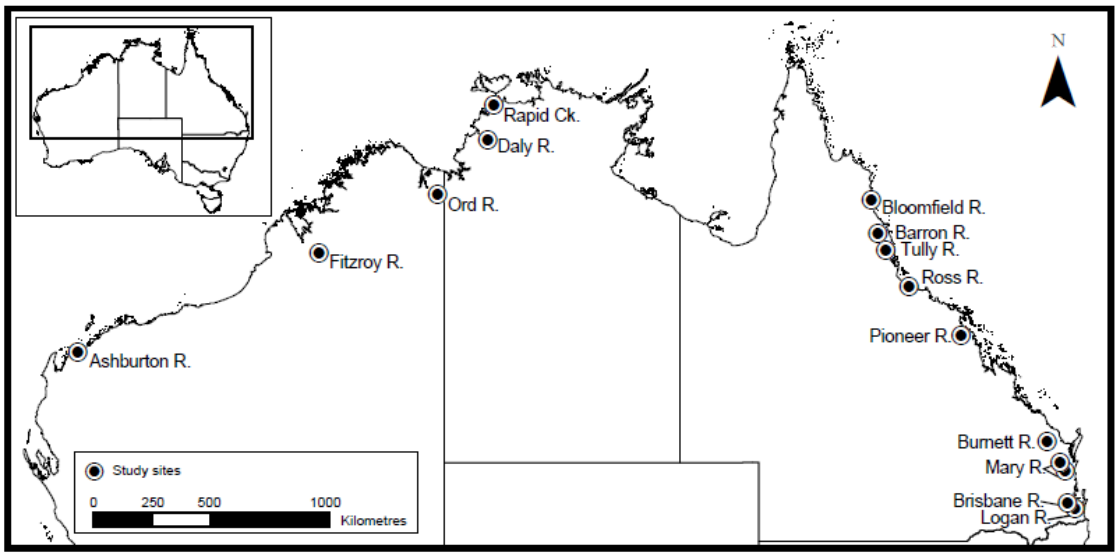

Figure 2. Location of sample sites in northern Australia.

Histopathology

For any fishes which tested positive for *E. ictaluri*, and for a subsample of fishes which tested negative, tissues were fixed in 10% buffered formalin for at least 24 hours, and processed using standard histology techniques. Formalin-fixed bony tissues were demineralised in 5% nitric acid for 1 hour before routine histo-processing and embedding in paraffin wax. Five micrometre sections were stained with haematoxylin and eosin for microscopic examination.

Data analysis

Fishes which tested positive for *E. ictaluri* by bacterial culture and DNA testing for either tissue sample (pooled kidney/spleen or intestine) were classed as infected. Prevalence of infection was calculated as the proportion of infected fish at a location, with 95% confidence intervals estimated using Jeffrey's method (Brown *et al.* 2001). This assumes a binomial distribution, which requires that each sampling event (i.e. each fish captured at a site) is independent and has the same probability that an infected fish will be found. These assumptions seem reasonable, given that our sampling methods rely on fish movement and there is no evidence that fishes without acute infections exhibit any different patterns of movement than uninfected fishes.

Results and Discussion

Detection of *E. ictaluri*

Edwardsiella ictaluri was detected in both pooled kidney/spleen tissue and intestinal tissue from eight *Tandanus tropicanus* sampled at one site; Bullyard Creek, a tributary of the Tully River in northern Queensland. All *E. ictaluri* isolates grew on blood agar as 0.3 mm colonies at 24 h and increased in size to 0.7 mm at 48 h with a slight greening of the agar. All isolates were positive for beta haemolysis, ornithine decarboxylase (ODC), lysine decarboxylase, reduction of nitrate, methyl red (MR), fermentation of glucose, maltose and mannose, and growth and motility at 24 and 37°C. Results were negative for arginine dihydrolase, Voges Proskaur reaction, urease, indole, citrate utilisation, aesculin hydrolysis, gelatin hydrolysis, ortho-nitrophenol and fermentation of arabinose, inositol, lactose, mannitol, salicin, sorbitol, sucrose, trehalose and xylose when tested in conventional biochemical media. Gas production was variable, with 44% of isolates positive for gas production when tested using a Durham tube in the glucose test. All isolates grew on deoxycholate agar and xylose deoxycholate agar, but not on brilliant green agar (BGA). These media are selective for *Salmonella* species.

Reports in the literature indicate some phenotypic variation among isolates of *E. ictaluri* from different geographic sites. Isolates from the USA and those previously detected in quarantined imports in Australia were reported as negative or had weak motility after growth at 37°C, were motile at 25°C, and grew more slowly at 37°C than 25°C (Hawke *et al*., 1981; Humphrey *et al*., 1986). Isolates from brown bullhead in the USA were negative for MR (Hawke *et al*., 1981), whereas most other isolates are positive. Growth has been reported negative at 2% NaCl, but isolates from the Tully River grew well at 3% NaCl when tested on agar containing 3% final NaCl concentration, and grew well, and were motile at both 25 and 37°C. Previous reports of growth on BGA medium was not found with the Tully River isolates or with an isolate previously detected in quarantined fish from Indonesia and isolated at Animal Health Laboratories, DAFWA. Isolates detected in Vietnamese catfish were negative for ODC (Crumlish *et al*. 2010), whereas Tully River isolates were positive for ODC. Most isolates, like those from the Tully River, are generally reported as negative for fermentation of mannitol, however isolates from rainbow trout cultured in Turkey were positive for mannitol when tested using conventional media and in the API20E kit (Biomerieux) (Keskin *et al*. 2004).

Disease ecology

Edwardsiella ictaluri infections were found in eight of 20 *T. tropicanus* sampled in the Tully River (Table 3; prevalence of infection 0.40, 95% confidence interval 0.21-0.61). Bacterial cultures of tissue samples from the 323 fishes collected from 14 other catchments throughout northern Australia were negative for *E. ictaluri*. Given the relatively large confidence intervals around the prevalence estimates (Table 3), we cannot confidently infer that the bacterium is not present at these localities. If the bacterium is present at one or more of these localities at our within-river design prevalence of 10%, then the binomial probability of capturing no infected fishes at any locality is 0.23. At this stage, therefore, all we can say with certainty is that *E. ictaluri* is present in one location, and either absent or at low prevalence elsewhere in Australia.

No fish (either infected or uninfected) from the Tully River exhibited any clinical signs, such as reduced movement, swimming in circles or hanging motionless in the water column, that have been associated with *E. ictaluri* infection in other studies (Hawke *et al.* 1998). There was no difference between infected and uninfected fish in either length ($t_{18} = 0.38$, $P = 0.71$), weight ($t_{18} = 0.07$, $P = 0.95$) or condition ($t_{18} = 1.25$, $P = 0.22$). Infected fish showed no gross abnormalities, either externally or on examination of internal organs. Histological examination of infected tissues found no evidence of pathological lesions associated with bacterial septicaemia.

Table 3. Number of fish sampled (N) and prevalence of infection (with 95% confidence intervals calculated assuming a binomial distribution) at each site.

Site ID	River	N	Prevalence	95% CI
BLR	Logan R	20	0	0-0.12
BBR	Brisbane R	20	0	0-0.12
TCM	Mary R	20	0	0-0.12
SPM	Mary R	20	0	0-0.12
BYB	Burnett R	20	0	0-0.12
MPR	Pioneer R	20	0	0-0.12
TRR	Ross R	16	0	0-0.14
CTU	Tully R	20	0.40	0.21-0.61
CBA	Barron R	20	0	0-0.12
CBI	Bloomfield R	19	0	0-0.12
DRC	Rapid Creek	30	0	0-0.08
NTD	Daly R	23	0	0-0.10
KLK	Ord R	27	0	0-0.09
KSC	Fitzroy R	20	0	0-0.12
PAR	Ashburton R	18	0	0-0.13

The Tully River system drains an area of 1,684 km^2 in the Terrain Natural Resource Management region of northern Queensland. To date, fishes at only one site within the system have been sampled for *E. ictaluri*. Although very little is known of the biology of the host species, *T. tropicanus*, studies on other Australian plotosid catfish species have found high site fidelity, with localised movements (< 2 km) for foraging, related to discharge levels (Reynolds 1983; Beatty *et al.* 2010; Koster *et al.* 2014). It is therefore possible that the infection is limited in extent within the Tully River system, although further sampling would be required to determine this.

Source of infection

With the present data, we cannot rule out the possibility that *E. ictaluri* is widespread in northern Australia at prevalence below the sensitivity of methods employed in this survey. If this is the case, then it may represent an endemic strain of the bacterium or an introduced strain that has been present for some time. There are some indications of phenotypic differences between *E. ictaluri* isolated from the Tully River and isolates from the USA and Vietnam, as well as those previously detected in quarantined imports in Australia. At present, however, phylogeographic data on strains of *E. ictaluri* are very limited, which precludes an accurate assessment of the origin of the bacterium.

If the presence of *E. ictaluri* in the Tully River results from a recent introduction, then the most probable source is through the accidental or deliberate release of an infected alien fish. The only alien species captured during sampling in the Tully River was the common platy, *Xiphophorus maculatus*.

This was also the only alien species recorded in the Tully River catchment by Kroon *et al.* (2015) and has been present in the river since at least 1994 (Hogan and Graham 1994). While neither *X. maculatus* nor any other poeciliid species have been recorded as known hosts of *E. ictaluri*, the bacterium appears to be a host generalist and there is no reason to believe that *X. maculatus* cannot act as a carrier. Alternatively, *E. ictaluri* may have been introduced into the Tully River by another alien fish species that has either not established breeding populations or is at such low density that it has not been detected. In addition to *X. maculatus*, five other alien fish species have been found in the Johnstone and Murray River catchments, adjacent to the Tully River catchment (Kroon *et al.* 2015); *Oreochromis mossambicus* (Mozambique tilapia), *Pelmatolapia mariae* (spotted tilapia), *Gambusia holbrooki* (mosquitofish), *Poecilia reticulata* (guppy) and *Xiphophorus hellerii* (green swordtail).

Management options

Edwardsiella ictaluri is able to survive for long periods (at least 95 days) in the benthos (Plumb and Quinlan 1986). Given the potential for survival outside of fish hosts, eradication of the bacterium is not a feasible control option. Any management actions which are undertaken should therefore be aimed at minimising the potential for spread from infected to uninfected rivers. As a first step, this requires an accurate estimation of the geographic distribution of *E. ictaluri* in Australia. The current survey, which was designed to determine the probability of disease freedom across the whole of northern Australia, did not have sufficient power to infer that individual rivers were free of disease.

Additional sampling to determine the geographic distribution of infection would need to consider, firstly, which rivers should be sampled and, secondly, the appropriate sample size to determine disease freedom. Targeted sampling requires an assessment of which rivers are most likely to contain infected fishes. If *E. ictaluri* is a non-native pathogen, then the most likely source of infection is through the release into rivers of infected alien fishes. In the current survey, we used proximity to human population centres as an indirect measure of the likelihood of a river containing alien fishes. This is clearly an imperfect measure, because the Tully River (where *E. ictaluri* was found) was designated low risk on the basis of the absence of major population centres, but still contained alien fish species. A more direct measure would be the documented presence of alien fish species, but this is largely limited to those with established breeding populations and is biased by sampling coverage (Corfield *et al.* 2008). In the absence of reliable data on the release of alien fishes, we believe that proximity to human population centres provides the best risk criterion for targeted sampling.

Once rivers are selected for sampling, an appropriate sample size to substantiate freedom from infection can be determined using either frequentist (e.g. Cameron and Baldock (1998) or Bayesian (e.g. Johnson *et al.* 2003) methodology. Both approaches require the determination of a particular threshold value of prevalence, below which the population can be considered to be disease free. For *E. ictaluri*, where fishes often remain infected after recovery and prevalence rates are typically high when the bacterium is present (Klesius 1992; Hassan *et al.* 2012), then a prevalence of 10% might represent an appropriate threshold value. With this threshold value, and the same assumptions for test sensitivity and specificity as employed in our survey, then a sample size of 36 fishes in a population of 1,000 would provide 95% confidence of disease freedom, using the approach of Cameron and Baldock (1998).

If further sampling determined with sufficient confidence that *E. ictaluri* is confined to the Tully River, then further management actions would depend on a risk assessment of the pathways by which the bacterium may spread, the probability of exposure of fishes following spread, and the consequences of infection to exposed fishes (OIE 2015). The most likely route for further spread of *E. ictaluri* is by the movement of infected fishes beyond the Tully River. This may occur through human agency, if infected fishes are removed and subsequently released in other catchments, or via natural movement of infected fishes via flood plumes along the coast or connectivity of floodplain wetlands. Karim *et al.* (2012) used a hydrodynamic model to investigate floodplain connectivity of the Tully and Murray Rivers for flood events of 1-, 20- and 50-year recurrence intervals. Connectivity was found even at the smallest recurrence interval, with duration of connection increasing up to 12 days as recurrence interval increased (i.e. with larger flood events).

Spread of *E. ictaluri* by sources other than infected fishes may also be possible. Taylor (1992) detected *E. ictaluri* in 53% of 137 piscivorous birds in the USA (snowy egret *Egretta thula*, great egret *Casmerodius albus*, great blue heron *Ardea herodius* and double-crested cormorant *Phalacrocorax auritus*), although most isolates could not be cultured. While the viability of the bacterium in birds is not known, the transfer of infection from the Tully River to neighbouring catchments through regurgitated crop contents or faeces cannot be ruled out. As *E. ictaluri* can survive in the benthos for extended periods of time (Plumb and Quinlan 1986), it is also possible that the bacterium could be transferred by mechanical means, for example on the feet of wading birds, or on boots, angling equipment or boats used by people.

Once moved into to a new catchment, the bacterium then needs to be transferred to uninfected fishes. The mode of transmission has not been definitively established, but is usually thought to be primarily from fish to fish via predation, scavenging or ingestion of bacteria shed in the faeces (Klesius 1992, 1994; Hawke *et al.* 1998; Xu *et al.* 2013). If *E. ictaluri* is spread by the movement of infected fishes, then these would need to come into contact with naïve fishes in the receiving catchment. There is also, however, circumstantial evidence that environmental sources of the bacterium (i.e. in the benthos) may be the major route of infection when acute disease is not present (Hassan *et al.* 2012), so fishes in the receiving catchment may be infected directly from transferred mud or water.

The consequences of infection to native fish species are difficult to predict. Australia has a unique freshwater fish fauna; of the 256 described species, 190 (74%) are endemic (Unmack 2013). This unique fauna is also highly imperilled, with 74 species (28%) listed as threatened under state or national legislation (Lintermans 2013). Introduced disease may therefore represent a significant additional threat to freshwater fish biodiversity. Very little information is available on the pathogenicity of *E. ictaluri* to native fish species, although clinical signs of disease were seen in the native catfishes *Anodontiglanis dahli*, *Neosilurus ater* and *Neoarius berneyi* that were found to be infected in an aquarium facility (Animal Health Australia 2012). In studies overseas, morbidity and mortality rates have varied widely, depending on fish species and environmental conditions (Buller 2015).

Other potential pathogens

In addition to *E. ictaluri*, 98 other bacterial species were detected in catfish samples throughout northern Australia (Appendix 2). While none of these were new occurrence records in Australia, there were a number of potential fish pathogens. A number of members of the Enterobacteriaceae family were isolated from the fish samples. These included *Aeromonas veronii* biovar Veronii, *Aeromonas hydrophila*, *Citrobacter freundii* and *Edwardsiella tarda*, *Vibrio cholera* non-01 and *Plesiomonas shigelloides*, all of which are opportunistic pathogens found in the environment and intestinal contents of fishes, amphibians, reptiles and some mammals. The presence of some of these bacteria, particularly *E. tarda*, seemed to be related to geographical origin of the fishes, but geographical origin is confounded with fish species in our study. *Vibrio cholerae* non-01 was isolated from intestinal contents on a few occasions. This has been reported to cause skin infection in ayu (*Plecoglossus altivelis*) and goldfish (*Carassius auratus*). It is commonly isolated in Australia from dams and freshwater sources. It can cause diarrhoea in humans and livestock. A number of other bacteria were isolated and are known saprophytes or members of the intestinal flora of fishes, including *A. veronii* biovar Sobria, *Vagococcus fluvialis*, *Lactococcus lactis*, *Lactobacillus* species, *Pseudomonas* species, *Shewanella putrefaciens* and *Acinetobacter* species.

Conclusion

A scenario tree method was used to design a risk-based survey for *E. ictaluri* in northern Australian native catfish. Catfish were sampled from 15 rivers across northern Australia, extending from the Logan River in Queensland to the Ashburton River in Western Australia. *Edwardsiella ictaluri* was detected at a prevalence of 0.40 (95% confidence interval 0.21-0.63) at one sampling site, in the Tully River in northern Queensland.

Implications

E. ictaluri was detected at one of the sites surveyed in this study. This may affect the export of native ornamental fishes from Australia to countries where the bacterium has not been found and provides information for the risk assessment for import of live fishes into Australia. Because the bacterium can survive in the bottom sediments of rivers, eradication is not a feasible option. If *E. ictaluri* is present only in the Tully River, it may be possible to minimise the potential for spread through appropriate management actions. While the survey did not find any evidence of infection at other sites, it is possible that it is present at low prevalence.

Recommendations

1) As an immediate, interim response, we recommend that actions be taken to minimise the risk of the spread of *E. ictaluri* from the Tully River to other localities in northern Australia. As the most likely route for further spread is by the movement of infected fishes, this may require further consideration of existing protocols for moving aquatic animals, and the provision of information about the bacterium and its potential modes of transmission to users of the river.

2) Recommendation (1) makes the provisional assumption that infection is confined to the Tully River. This has not yet been established. To determine with more accuracy whether *E. ictaluri* is isolated to the one locality at which it was detected or is more widespread throughout northern Australia will require additional sampling. Assuming that *E. ictaluri* is an introduced pathogen, this should be targeted at localities which are in close proximity or downstream of human population centres as these are most likely to have been exposed to escaped alien fishes. Sample size should be sufficient to confidently reject the null hypothesis that the locality contains infected fishes at a designated threshold prevalence.

3) If *E. ictaluri* is widespread, then the only management actions required may be to passively monitor fish health in affected rivers. If the bacterium is localised to the Tully River, then a risk assessment would be required to determine whether additional management activities are required. There are some key information gaps that need to be filled before an effective risk assessment can be undertaken. Of particular importance is the susceptibility and tolerance to infection by *E. ictaluri* of Australian native fish species, and how these may be influenced by external environmental conditions such as temperature and by endogenous factors such as immunocompetence and stress response.

Extension and Adoption

At the commencement of the project, a media statement detailing the aims of the survey was released on the Murdoch University website. This led to a number of media reports (detailed below).

Upon confirmation of the presence of *E. ictaluri* in wild catfish, the Queensland Department of Agriculture, Fisheries and Forestry and the Australian Government Department of Agriculture were informed of the finding and the location of infected fish.

The catfish specimens which were collected during the survey were preserved following tissue collection and have been subsequently used by Erin Kelly in an MSc research project at Murdoch University on the health of native catfish populations in northern Australia.

Project coverage

Newspapers

Kimberley Echo 20 March 2014: *Freshwater bacterium harmful to our fish.*

Broome Advertiser 31 March 2014: *Study probes catfish for disease.*

Websites

Science Network WA: http://sciencewa.net.au/topics/fisheries-a-water/item/2704-survey-set-to-prove-northern-fish-disease-free/2704-survey-set-to-prove-northern-fish-disease-free

PhysOrg: http://phys.org/news/2014-03-survey-northern-fish-disease-free.html

FishChannel: http://www.fishchannel.com/fish-news/2014/01/australia-testing-catfish-for-exotic-disease.aspx

Aquaculture Direct: http://aquaculturedirect.blogspot.com.au/2014/01/new-survey-to-test-catfish-for-exotic.html

The Fish Site: http://www.thefishsite.com/fishnews/22286/new-survey-to-test-catfish-for-exotic-fish-diseases/

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Appendix 1: Survey design for detection of *E. ictaluri* in wild catfish populations

Freedom from infection

It is never possible to *prove* that a population is free from a pathogen. This would require simultaneous application of a perfect test to the entire population, and the testing would need to be repeated continuously. It is therefore necessary to take a probabilistic approach, and estimate the probability that the population is free from infection, given the results of a survey or ongoing surveillance process. The approach commonly used in terrestrial and aquatic animal health is that outlined by Martin *et al.* (2007), and this is the method we are using here.

Evidence for the absence of a pathogen from a population is acquired by failing to find it when we look for it. If the process used to detect the pathogen is highly sensitive (i.e. there is a high probability of detecting the pathogen when it is present) and we do not detect it, then we can say with confidence that it is not present. On the other hand, if our detection process has only low sensitivity (there is only a low probability that we will detect it when it is present) then failure to detect the pathogen does not give us much confidence that it is not present. This applies at all levels – in this case, to the test applied to the individual fish; the testing done at a particular sampling site on a river; all the testing done on the river system; and the whole set of testing done across Northern Australia.

In designing a survey aimed to deliver confidence in the absence of the pathogen from the population, we must aim at ensuring the survey has high sensitivity (Survey Sensitivity; *SSe*). Our confidence in the population being free from the pathogen, or the probability that the population is free from the pathogen given negative survey results (*pFree*), is then given by

$$pFree = \frac{1 - PriorPInf}{1 - PriorPInf \times SSe} \quad (1)$$

where *PriorPInf* is the prior (pre-survey) probability that the population was infected with the pathogen (Martin *et al.* 2007). In a scenario such as that in question here (presence/absence of *E.ictaluri* in Northern Australian native catfish) *PriorPInf* is unknown, and there is very little information on which to base an estimate; it is then appropriate to assign it a value such as 0.5. Use of the approach represented by Equation 1 requires a number of further procedural assumptions:

1. Calculation/estimation of *SSe* (the probability of detecting infection *given that it is present*) assumes that the population *is infected*. The (hypothetical) level of infection in the population is given by one or more *design prevalence(s)*, which are arbitrarily specified parameters of the survey. Then, having calculated *SSe* (after the survey), which we can then consider to be the probability that we would have detected disease were it present, we use Equation 1 to assess the probability that it was indeed present at the time we conducted the survey. From this it is clear that both *pFree* and *PriorPInf* refer to freedom from, and presence of, infection *at the design prevalence(s)*.
2. The survey has perfect specificity; *ie* there is no chance of declaring the population infected when it is actually not infected. In matters relating to international trade this is a robust assumption (Martin *et al.* 2007), and in designing this survey we make the same assumption. It is believed that *E.ictaluri* is not present in Australia, so any positive test result will be followed up with repeated culturing, application of other tests, further sampling at and around the site from which the suspect sample came, etc. At the end of these follow-up procedures an unambiguous conclusion will be arrived at – *E. ictaluri* was present in that fish, or it was not. The overall survey may then be said to have perfect specificity, even though the laboratory test applied to the individual sample may not be perfectly specific.

Risk-based sampling

When looking for something, we have the greatest chance of finding it if we look in places where it is most likely to be. In a survey such as this, we have a greater chance of detecting the pathogen if we focus our sampling on those sites at which it is more likely to be present. In other words our survey will have higher sensitivity if we sample high-risk fish. This concept flies in the face of traditional cross-sectional survey design, which requires representative sampling of the population. However, the methodology of Martin *et al.* (2007) makes possible quantification of the sensitivity of risk-based, non-random sampling strategies. In fact, the fundamental principle of representative sampling is retained, but the population is first stratified by all factors affecting the probabilities of infection and detection, and representative sampling is required within each of the resulting strata. Care must be taken, however, that significant sub-populations are not ignored in the resulting sampling program (designed to maximise *SSe*), which might ignore, for example, all waterways in a particular region or State.

In this survey we aim to estimate the probability that northern Australia is free from *E. ictaluri* infection in native catfish, based on sampling fish primarily at high-risk locations.

Populations at risk

To date, all reports of *E. ictaluri* in Australia have been associated with imported fish or aquarium facilities. In the absence of an import risk analysis, we therefore assume that the most likely source of infection to wild fishes is through the accidental or deliberate release of infected aquarium fishes. Other potential mechanisms for introduction (e.g. birds, visiting boats) have not been considered.

There are no data on the release of aquarium fishes in Australian rivers, and although there are some data on the number of established species (i.e. those with breeding populations), these are biased by sampling coverage (Corfield *et al.* 2008). In determining populations of wild fish at most risk of infection, therefore, we have used proximity to human population centres as the initial criterion. There is a strong positive relationship between population size of a city or town in Australia and the number of aquarium shops (Figure A1).

Because *E. ictaluri* infections have not been found at water temperatures below 18ºC and acute ESC usually occurs between 20-30ºC we have restricted our investigation to populations of catfish in tropical and sub-tropical northern Australia (i.e. Queensland, Northern Territory and the Kimberley and Pilbara regions of Western Australia).

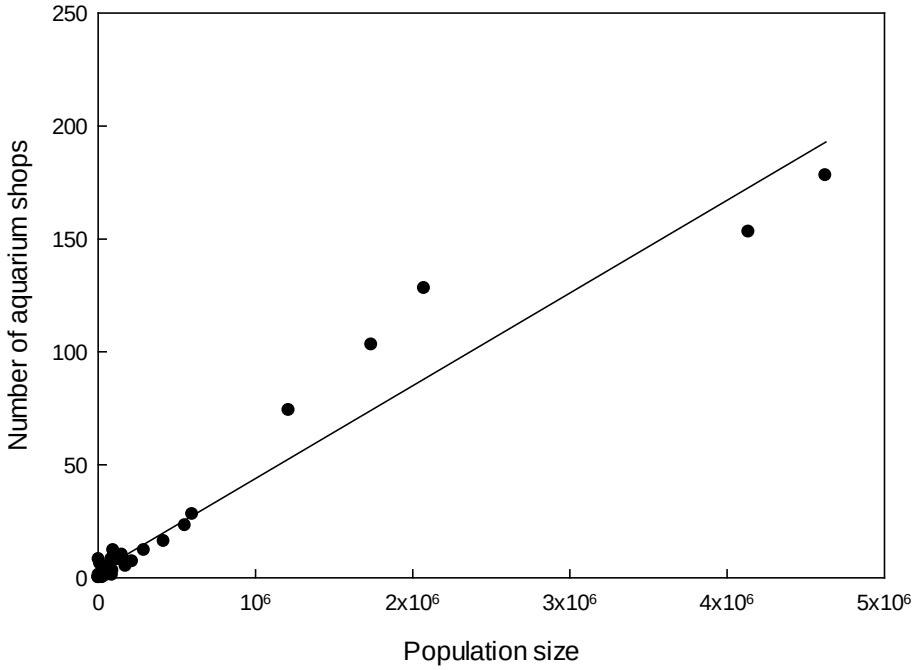

Figure A1. Populations (x) of towns and numbers of aquarium shops (y), determined from Australian yellow pages search. Populations <1,000 excluded. Significant linear regression; $r^2 = 0.93$; $F = 914.7$, $P < 0.0001$). $y = 2.13 + (3.95 \times 10^{-5}) x$.

Diagnosis of infection

Available diagnostic techniques include bacterial isolation by culturing, ELISA, IFAT and PCR tests (OIE 2009). Selective culturing of homogenised kidney tissue culture has been found to be the most reliable method of determining infection status in both clinical and subclinical infections, with a diagnostic sensitivity and specificity of 0.92 and 0.91 respectively (Bebak *et al.* 2011). Although PCR has a higher diagnostic specificity of 0.95, it has a diagnostic sensitivity of only 0.43 (Bebak *et al.* 2011). These authors determined diagnostic sensitivity and specificity of these tests by reference to a *gold standard* test – in this case broth culture of homogenised tissue in serial dilutions. A specificity of 91% for a test involving culture of a readily characterised organism suggests strongly that the reference test is itself flawed, as they acknowledge in their discussion.

Scenario tree model

Our survey design tool is a model of the survey process which allows us to estimate *SSe*, the sensitivity of the survey. The model defines all ways in which a positive outcome (isolation of *E.ictaluri*) can be obtained from a population infected at the design prevalence(s). The model is represented diagrammatically using a scenario- (or event-) tree defining the logical sequence of steps involved in obtaining a positive outcome, and thus the parameters/variables required to estimate the probability of a positive outcome. Such a diagram is shown in Figure A2, which shows a series of nodes (branching points) in a hierarchical structure. The starting point (population infected with *E.ictaluri*) is at the top; in order to obtain a positive outcome in the survey (red triangle at the bottom) one of the pathways shown through the various nodes must be followed. The two *category nodes* in the diagram (TOWN(S) PRESENT ON RIVER SYSTEM and LOCATION OF SAMPLING SITE) subdivide the population into multiple categories, and the same node structure as that shown applies to each of the branches; only the 'limbs' of the 'tree' which represent fish caught *Within townsite*s are shown completed, in order to keep the diagram minimally cluttered.

This is a model of all fish tested in the survey. The 'node' FISH IS CAUGHT is included both to emphasise this point, and to illustrate the relationship between this and the following infection node

FISH IS INFECTED (see below); although since FISH IS CAUGHT has only one branch, with a probability of 1, its presence is unnecessary.

Each branch of a category node has an associated branch proportion (of the population or subpopulation being categorised by the node), and each branch of an infection node or a detection node has an associated branch probability. Branch proportions or probabilities sum to one for each node. All probabilities and proportions are conditional on preceding (higher level) nodes in the tree; so in Figure A2 the probability that a fish is infected (*Yes* branch of FISH IS INFECTED node) is that for a fish caught within a townsite on an infected river system. The probability that a single fish tested in this survey will yield a positive outcome is given by multiplying together all the branch probabilities (infection and detection probabilities) applicable to the limb on which the fish lies. For a fish randomly selected from those tested in the survey (i.e. one for which we don't know the river system or sampling location) the probability of a positive outcome is the sum of the limb probabilities for all six limbs with positive outcomes, where a limb probability is the product of all proportions and probabilities for the branches comprising the limb.

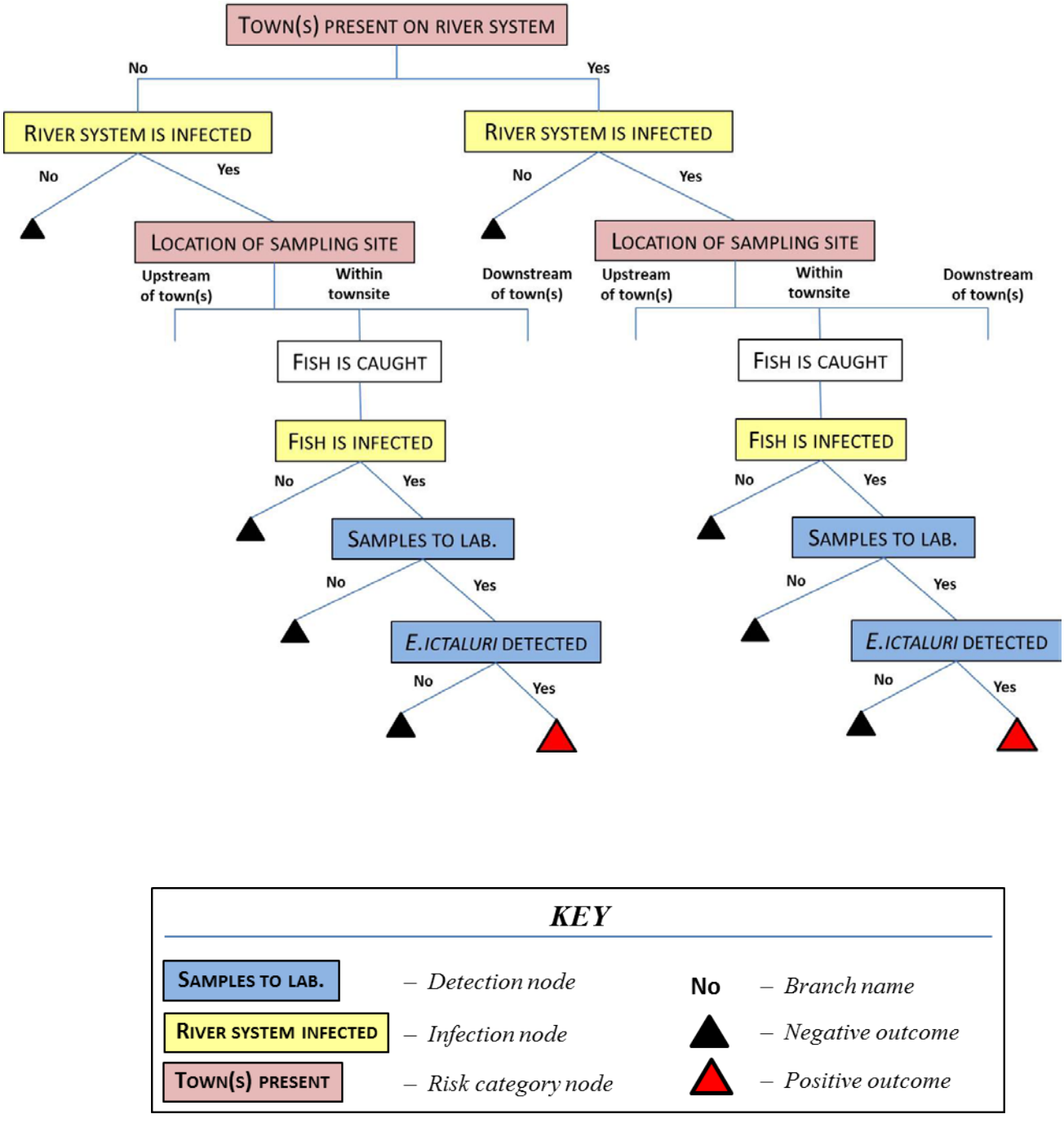

Figure A2. Scenario tree model of survey process.

Reference population

The population of wild catfish to be surveyed is that found in northern Australia, specifically:

- The Kimberley and Pilbara regions of Western Australia;
- Northern Territory; and
- Queensland south to Brisbane.

Twenty species of catfish from two families (Ariidae and Plotosidae) have been described in Australia and at least 15 of these species are found in northern Australia. All wild catfish species are included in the survey.

Sub-populations and risk factors for infection

The population is divided into a series of river systems, each of which is distinct; separated by catchment boundaries. There are 55 (*NumRivers*) major river systems in Northern Australia; they are listed in Table A1, which includes 2 potentially high risk creeks adjacent to Darwin.

Table A1. Major river systems in Northern Australia (Queensland, Northern Territory and Kimberley and Pilbara regions of Western Australia) considered for sampling.

Region	Number of rivers	River names
Queensland	23	Logan, Brisbane, Maroochy, Burnett, Boyne, Dawson/Fitzroy, Burdekin, Ross, Pioneer, Tully, Mulgrave, Barron, Daintree, Jardine, Archer, Mitchell, Gilbert, Norman, Flinders/Saxby, Leichhardt, Nicholson/Gregory, Mary, Bloomfield
Northern Territory	17	Robinson, McArthur, Limmen Bight, Roper, Goyder, East Alligator, West Alligator, South Alligator, Mary, Adelaide, Finnis, Daly, Victoria, Elizabeth, Rapid Ck., Ludmilla Ck.
Kimberley	10	Ord/Bow, Pentecost/Durack, Drysdale, King Edward/Carson, Mitchell, Roe, Prince Regent, Isdell, Meda, Fitzroy/Hann
Pilbara	5	De Grey, Fortescue/Robe, Ashburton, Gascoyne, Murchison

Spread of infection among river systems could potentially occur during flooding, but apart from such considerations the river system effectively represents an isolated sub-population of catfish whose infection status is independent of the infection status of other river systems. Within a river system we assume that it is possible for infection to spread without hindrance. Based on this conceptual model (clustering of infection in river systems) we have allocated two design prevalences to describe the (hypothetical) level of *E. ictaluri* infection in the population:

- among-river design prevalence (P_R^*) and
- within-river design prevalence (P_F^*).

For calculation of *SSe* we assume the population is infected such that the proportion P_R^* of river systems is infected, and within an infected river system the proportion of fish infected is P_F^*. Overall, the proportion of fish infected is then $P_R^* \times P_F^*$.

Some rivers are more likely to be infected than others, and within a river some fish are more likely to be infected than others. Risk factors for infection that we have identified are detailed below.

- ***River system infection*** The presence of one or more town(s) on a river system (represented by the risk node TOWN(S) PRESENT in Figure 2. Risk is proportional to human population.

Other potential among-river risk factors which we considered, but for which we could find no supporting evidence, include:

- pollution;
- human activity on the river (other than dumping of aquarium fish);
- soil types; pH; mineral content;
- presence of other species in or around the river.

- ***Within-river infection*** The location of fish within the river system relative to any town that may be present (LOCATION OF SAMPLING SITE) in Figure A2. Three levels of risk are specified:
  - *Upstream* of any towns on the river (lowest risk);
  - *Downstream* of any towns on the river. Since substantial towns (population >5,000) are generally on or close to the coast, and therefore the river mouth, the proportion of a river system falling into this category is generally very low;
  - Within *Townsite* (highest risk). The boundaries of a townsite are defined by the estimated distance (*TownRadius*) that people might travel to dump aquarium fish in a waterway.

  Other potential within-river risk factors for infection of fish with *E. ictaluri* which were considered, but for which no supporting evidence could be found, include:

  - species;
  - age;
  - sex.

Risks are applied in the model as *relative risks* – for each risk category, the risk relative to the lowest risk category. For LOCATION OF SAMPLING SITE within an infected river system, this is straightforward – we simply specify risks for *Downstream* and *Townsite* relative to *Upstream*, which takes the value of 1. While it may be simple to specify such relative risks, estimating 'correct' values is tricky, since in a disease-free population there are no data on which to base the estimate. We rely on the opinions of the research team following review of any available relevant literature, and then evaluate the sensitivity of the outcome to variation of the selected relative risks.

Relative risks (RRs) for infection of river systems were estimated for each river system individually, using a linear scale with maximum among-river RR (*MaxARRR*) allocated to the river system with the highest aggregate human population of towns on its river(s), among all *NumRivers* systems (*MaxPop*), and minimum RR (*MinARRR*) for a river system with a town allocated to the system(s) with a population of 5000.

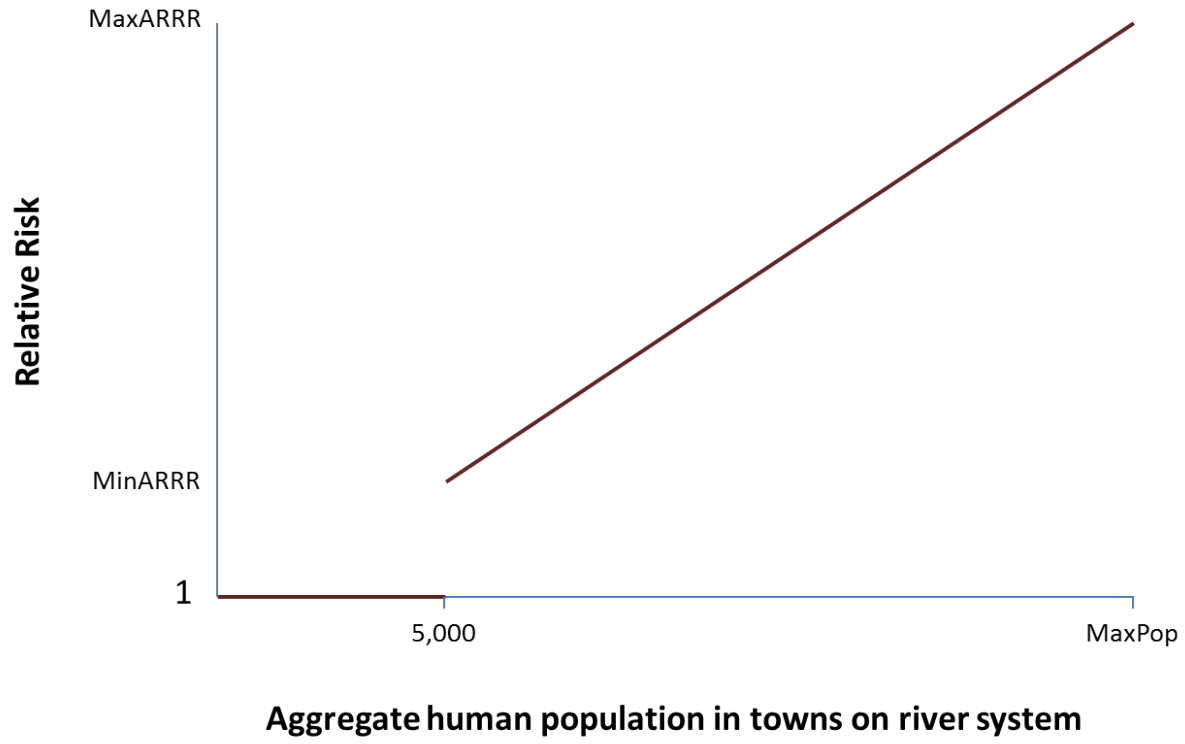

Figure A3. River system relative risks.

RRs are combined multiplicatively with design prevalence to give the Effective Probability of Infection (EPI) for each River (EPIR), or each Fish within an infected river (EPIF). However, in order to ensure that the overall, average, probabilities of infection for river systems, and for fish within an infected river system, are equal to the specified design prevalences (P_R^* and P_F^*), the RRs (which are necessarily greater than 1) must be adjusted or 'normalised' so that they average 1 over the reference population of rivers (and fish within an infected river), while maintaining their specified relativities. The mechanism for doing this is given by Martin *et al.* (2007). For a 3-category risk node such as LOCATION OF SAMPLING SITE with categories 1, 2 and 3, the RRs are RR_1, RR_2 and RR_3. The proportions of the reference population represented by this node (in Figure 2, the proportions of river system 7) falling into each category are PrP_1, PrP_2 and PrP_3. The adjusted (or normalised) risk for category i (AR_i) is then given by

$$AR_i = \frac{RR_i}{\sum_{i=1}^{3}\left(RR_i \times PrP_i\right)} \tag{2}$$

Then

$$EPIF_i = AR_i \times P_F^* \tag{3}$$

It is apparent from Equation 3 that RR_i must be constrained such that $EPIF_i$ cannot exceed 1. This is straightforward as long as values chosen for RR_i are restricted to what is logically possible; it is not possible, for example, for a small population proportion to have a large RR when the design prevalence is high. Suppose a *townsite* occupies 1% of a river system, and P_F^* is 0.1. This means that infected fish will occupy 10% of the river system (assuming, as we are, that fish are evenly distributed throughout the river system). If we now say that *townsite* fish are 100 times more likely than either upstream or downstream fish to be infected, we are requiring around 99% of the infection in the river to be present in 1% of the river, while in fact it requires 10% of the river. This is therefore illogical and invalid; the maximum possible AR value is equal to $1/P_F^*$.

Adjustment of RRs for TOWN(S) PRESENT ON RIVER SYSTEM is done similarly, except that each river system forms its own category, and has a standard PrP of 1/*NumRivers*; and each river system's RR is calculated individually as described above (Figure A3).

Sampling the population

Homogenised kidney tissue is required for laboratory culture of *E.ictaluri*. Fish collected for tissue samples must be caught by the selected method to ensure consistency in sampling bias across all sites. . Are caught fish more (or less) likely to be infected than fish that are not caught? The nets to be used will catch actively swimming fish. Sick fish are less likely to be mobile, so are less likely to be caught; this will apply primarily to chronically infected fish. Recovered, carrier fish are likely to be as active as uninfected fish. We have included a variable allowing an appropriate adjustment of the probability that a caught fish is infected; *RRCaught_Inf*, the Relative Risk of being caught for an infected fish (relative to an uninfected fish). Since infection status precedes being caught, we estimate values for the probability that an uninfected fish will be caught (*pCaught_Uninf*) and the probability that an infected fish will be caught (*pCaught_Inf = pCaught_Uninf* × *RRCaught_Inf*). A straightforward application of Bayes theorem then gives the Effective Probability of Infection for a Caught Fish (*EPICF*):

$$EPICF = \frac{EPIF \times pCaught\_Inf}{EPIF \times pCaught\_Inf + (1 - EPIF) \times pCaught\_Uninf} \quad (4)$$

This is the value used for the probability of the *Yes* branch of the FISH IS INFECTED node of Figure 2.

Detection probabilities

The detection node SAMPLES TO LAB. is included in the model diagram (Figure 2) simply to show that its scope has been considered. For the purposes of survey design we assumed that the probability of appropriate samples being correctly collected, prepared and transported to the laboratory in good condition is 1. In practice some samples may either not arrive at the laboratory, or arrive in poor condition, or be mishandled in the laboratory; in such cases they will not be processed, so the chance of a positive outcome will be zero anyway.

Thus the only variable in this survey that affects the probability of a positive outcome for an infected processed fish is the probability that *E. ictaluri* is detected in the laboratory. Given appropriate samples, the sensitivity of kidney culture (*TestSe*) can be estimated from published results.

Calculation of survey sensitivity

River system sensitivity (*SeR*; the probability that an infected river system will give a positive outcome given the testing done on that river) is given by

$$SeR_k = 1 - \prod_{j=1}^{J_k} \left(1 - TestSe \times EPICF_{j,k}\right)^{n_{j,k}} \quad (5)$$

where $EPICF_{j,k}$ is the effective probability of infection for a caught fish taken at sampling site *j* on river system *k*; $n_{j,k}$ is the number of fish sampled at site *j* on river system *k*; and J_k is the number of sampling sites on river system *k*. Equation 5 assumes that the fish taken from the river at a sampling site are representative of a large number of fish (>10 × n_j) present at the site, and that there are many (>10 × J_k) potential sampling sites on the river. We believe these are valid assumptions, so the use of a binomial probability formula is appropriate.

Overall survey sensitivity is then

$$SSe = 1 - \left(1 - \frac{nR}{NumRivers} \times AveSeR\right)^{AveEPIR \times NumRivers} \quad (6)$$

where *nR* is the number of river systems from which samples are taken; *AveSeR* is the average *SeR* for rivers from which samples are taken, and *AveEPIR* is the average *EPIR* for rivers from which samples are taken. Equation 6 gives an approximation of the hypergeometric probability that one or more river

will give a positive outcome; it is necessary to use this formula because a large proportion (>10%) of rivers will be sampled.

Modelling uncertainty

Various parameters for this model must be estimated in the absence of good data. Where appropriate we have modelled our uncertainty about the values assigned to those parameters using Pert distributions, allowing simulation of a stochastic model for analysis of the results. In estimating an optimum sampling strategy we used the expected value (mean) of each of these distributions. The model is implemented in MS Excel 2010 with the PopTools Add-in.

Determining an optimum sampling strategy

Having set up the model for calculating *SSe* and *pFree* we determined the desired value for *pFree* and set appropriate design prevalences. Using the model we then determined a number of river systems to sample that gave a reasonable level of confidence in freedom (given a negative survey outcome) and adjusted numbers of sampling sites and numbers of fish to be taken at each site to give the desired *pFree*. We then explored the sensitivity of the model outputs to values specified for relative risks, adjusting them where necessary to ensure that we were not assigning unsupportable levels of risk which had a substantial impact on the survey outputs. The end result is clearly one of many possible sampling strategies, and can be adjusted "on the run" to achieve comparable survey outputs.

Selection of values for model inputs

Risk nodes

The largest town in the study area is Brisbane, with a population over 10 times that of the next largest. Consequently the value given to *MaxARRR* can potentially be very influential, and in many scenarios sampling 5 fish in Brisbane alone can yield an *SSe* of over 50%. This does not make it representative of the population, however. Values chosen for design prevalences are crucial. If the survey is to be truly representative of the reference population it is unreasonable to sample only in highest-risk sites. At the same time, given the underlying premise that if infection is present it will have been introduced by the dumping of aquarium fish in or near towns large enough to have suppliers of aquarium fish and equipment, it makes good sense to ensure that high risk sites are all included in the survey.

Town(s) present on river system

The highest aggregate population of towns >5,000 on a river system is 2,086,524, for the Logan River. *MaxARRR* was given the value(s) shown in Table A3, which then applied to the Logan River, with other rivers with towns allocated a RR on a linear scale down to *MinARRR*, which applied to the Ord/Bow and Daintree river systems, both of which have aggregate populations of around 5,000. Values of 3 (*MinARRR*) and 10 (*MaxARRR*) were selected for the range of among-river risks for the following reasons.

- With around 50 river systems and an among-river design prevalence of over 10% (see below for the reasons for this), around 10 is the highest value the RR for a river can logically take.
- Since we assume the native catfish population to be naturally free of *E.ictaluri*, the risk of infection for a river with one or more towns present must be substantially higher; twice as high does not seem enough, given the mechanism for introduction of infection that is assumed.
- As potential sources of infection for uninfected river systems, the risk associated with a town must increase with increasing population, and given the wide range of human populations involved (largest is 400 times the size of the smallest), the range of risks must be as wide as possible. In addition, large city dwellers are more urbanised in their outlook, and probably less aware of the potential consequences of irresponsible disposal of unwanted, or even sick, fish than their small-town counterparts.

Location of sampling site

When infection has been introduced at a townsite (and in our model it has, in $1 / P_R^*$ rivers), will it have spread from the townsite? Upstream? Downstream? Fish will move either up or down depending on the location of most suitable habitat; bacteria free in the water will spread downstream, although this is not thought to be an important means of spread of infection; bacteria in the benthos can survive for months, but may not be carried far downstream. RRs are not easy to estimate here, and we used values of 1 (Upstream), 3 (Downstream) and 5 (Townsite) as defaults. Model outputs were not particularly sensitive to the values used, as long as we have them in the right order (Townsite > Downstream > Upstream).

The list of potential sites used in this analysis of sampling options is as shown in Table A2. The list is not comprehensive, and may be changed when sites are finalised. The order in which sites are listed is important, since as the number of sites to be sampled is adjusted, the specified number is taken from the top of the list. Sites 1 to 5 are high-risk (towns), while 6 – 10 are low-risk (rivers with no towns >5,000). This ensures that the likely minimum number of sampling sites (10) will include remote, low risk rivers so that 'townless' river systems will be represented in the survey. Sites 11 to 15 are then high-risk (towns), and 16 to 30 are low-risk. Estimated river lengths are included in Table A2 only for those rivers with towns of population >5,000, since these figures are only required for calculation of within-river population proportions for the LOCATION OF SAMPLING SITE node, which is only relevant where there are one or more towns on the river. For rivers where there are none, there is no within-river differential risk, and sampling site RR is 1 throughout the whole river system. That said, on townless river systems we will probably choose downstream sampling sites for convenience.

Table A2. Potential sampling sites used in modelling.

Site	Site name	River name	Aggregate urban population for river system	River length (km)
1	Brisbane	Brisbane	2,074,000	344
2	Townsville	Ross	176,000	50
3	Cairns	Mulgrave	153,000	100
4	Darwin	Rapid Ck	128,000	10
5	Kununurra	Ord/Bow	5,000	1,355
6	Bloomfield R.	Bloomfield	0	
7	Drysdale R.	Drysdale	0	
8	Fitzroy/Hann R.	Fitzroy/Hann	0	
9	Tully R.	Tully	0	
10	Adelaide R.	Adelaide	0	
11	Bundaberg	Burnett	69,500	500
12	Rockhampton	Dawson/Fitzroy	78,500	1,000
13	Brisbane S	Logan	2,086,524	150
14	Maryborough	Mary	21,500	250
15	Gympie	Mary	18,600	250
16	Daly R	Daly		
17	Ashburton R	Ashburton	0	
18	Mackay	Pioneer	120,000	120
19	Cairns N	Barron	153,000	150
20	Roper R.	Roper	0	
21	Prince Regent R.	Prince Regent	0	
22	Mitchell R.	Mitchell	0	
23	Robinson R.	Robinson	0	
24	East Alligator R.	East Alligator	0	
25	Maroochy R.	Maroochy	0	
26	Boyne R.	Boyne	0	
27	Burdekin R.	Burdekin	0	
28	Jardine R.	Jardine	0	
29	Norman R.	Norman	0	
30	Leichhardt R.	Leichhardt	0	
31	Archer R.	Archer	0	

Design prevalences

The ideal survey outcome might be 95% (or greater) confidence that no rivers are infected. This requires a survey with a 95% chance of detecting a single infected river, for which all rivers must be tested using a sufficient level of sampling to be similarly confident of detecting infection in each river. The among-river design prevalence would be 1 / *NumRivers* = 0.021. What would be the prevalence of infected fish be in an infected river? Perhaps no more than 5%, so the within-river design prevalence would need to be no more than 0.05. Such a survey would then require testing of 70 or so samples from each river, although you would get away with many fewer in high-risk rivers (as low as 20 in some). The total number of sampling sites would be at least 47, and 70 samples from each would mean 3,290 homogenised catfish livers. Our proposal budgeted for sample numbers more in the region of 150-300, and this figure underlies the scenarios presented here.

Assuming that most or all highest risk river systems, and high risk townsites within them, are sampled, there will be ten or so high-risk rivers sampled. The case for sampling a number of low risk rivers to improve coverage of the population of river systems has been presented, and if five of these are sampled there will then be a minimum of fifteen rivers sampled. Ten samples from each sampling site gives a total of 150 samples to process. Appropriate design prevalences to use for approximately this number of samples are P_R^* = 0.1 and P_F^* = 0.1. The survey will then aim to detect infection if it is present in 5 or more Northern Australian river systems, at a prevalence of 10% of fish in an infected river system, with a confidence of 95%.

Sensitivity of laboratory test

Bebak *et al.* (2007) assessed the sensitivity of culturing homogenised kidney tissue using experimentally infected fish. Many of these died from acute infections with *E. ictaluri*. In these circumstances, the *TestSe* was 0.92 (95% CI = 0.89, 0.96). The authors point out, however, that if the test is to be used on subclinically infected and carrier fish, it might be more realistic to use the sensitivity of the test as applied to samples containing $<10^4$ colony-forming units per gram of tissue. Our scenario is unlikely to involve any acutely infected catfish, so we are using the more conservative, lower sensitivity of 0.80 (0.71, 0.89), represented in the model using a beta distribution.

Risk of an infected fish being caught relative to that of an uninfected fish

We estimate this to be close to, but marginally less than, 1. We have used a Pert distribution with minimum possible value 0.9, most likely 0.95, and maximum possible 1.0.

Results

A selection of possible sampling strategies is presented in Table A3. Each of these delivers a survey sensitivity of around 95% and a probability of population freedom from *E. ictaluri* of 95%. The among-river design prevalence in this table is presented as its reciprocal, which is the number of (hypothetically) infected river systems in the population.

In comparing these different options, the following points are of interest.

- The only option presented which keeps sample numbers around 150 is A, for which P_R^* is 6/*NumRivers* (six river systems are infected).
- Options E and F are for a sampling system which is not risk-based – there is no benefit in terms of sensitivity gained from sampling townsites over other sites. More samples per site and more sampling sites are required to generate the same level of *SSe* as the risk-based sampling schemes.
- The number of samples collected at each sampling site is a standard across-the-board figure in each of these scenarios. However, different numbers are needed at different sites to achieve similar levels of sensitivity – less are needed from higher risk sites. When a list of sampling sites has been settled on, site-specific sample numbers can be generated which will allow minimising of overall sample numbers to achieve the end result.
- As the number of sites increases past 15, the marginal benefit of sampling more sites decreases, for two reasons:
- This is how it is with all such sampling programs for demonstrating freedom from disease – the more negative samples you accumulate, the less the marginal benefit. This effect also applies to the number of samples taken at each site.
- Assuming (as this model does) that only one site is selected per river system, all sites after number 15 will be low-risk.

Table A3. Sampling numbers and other model inputs to deliver *pFree* of 95%[2] showing six alternative approaches.

Input	Option A	B	C	D	E	F
P_F^*	**0.1**	**0.05**	**0.1**	**0.1**	**0.1**	**0.1**
$1 / P_R^*$ [1]	**6**	**9**	**5**	**5**	**5**	**5**
Samples per site	**11**	**11**	**18**	**11**	**37**	**20**
No. of sampling sites	**15**	**15**	**15**	**25**	**23**	**28**
TestSe	0.79	0.79	0.79	0.79	0.79	0.79
PriorPInf	0.5	0.5	0.5	0.5	0.5	0.5
NumRivers	53	53	53	53	53	53
TownRadius (km)	30	30	30	30	30	30
Among-River RRs						
No town present	1	1	1	1	1	1
MinARRR	3	3	3	3	1	1
MaxARRR	10	9	10	10	1	1
Within-river RRs						
Upstream of town(s)	1	1	1	1	1	1
Downstream of town	3	3	3	3	1	1
Townsite	5	5	5	5	1	1
RRCaught_Inf	0.95	0.95	0.95	0.95	0.95	0.95

[1] The reciprocal of the among-river design prevalence, which is the number of river systems that are (hypothetically) infected.

[2] *SSe* was also 94% - 95% for all options.

Figures in **bold** are critical values with significant impact on the survey sensitivity and confidence in freedom derived from a negative survey outcome

1. Comparing options A and B shows the interplay of P_F^* and P_R^* in this model. Decrease one, and you must increase the other to maintain *SSe* with the same sample numbers.
2. Options C and D give two approaches to sampling at the selected design prevalences. C requires 270 samples in total, while D needs 275 – these numbers are well balanced because the numbers of samples per site is sensibly low, and marginal benefit of more samples at a site is significant. It is likely that 25 sites on 25 rivers will be too difficult to achieve in practical and financial terms, although sampling 25 rivers would look more convincing than sampling 15. On the other hand, taking only 11 samples at each of 25 sites gives an average river-level sensitivity (*SeR*) of 0.66; hardly high enough to make confident statements about the infection status of any individual river. Average *SeR* when 18 samples are taken from each of 15 river systems is 0.88.
3. The reason for specifying 37 samples per site in option E is to illustrate the number needed to give an average *SeR* of 0.95; in other words, giving us an average of 95% confidence that we will detect infection in each of the 23 rivers sampled (it will be higher for high-risk (town) sites and lower for sites on townless rivers).

4. In option B, P_R^* had to be raised to 9/*NumRivers* to retain the sampling numbers of option A when P_F^* was reduced to 0.05. The higher P_R^* rendered a *MaxARRR* of 10 untenable so we had to reduce it to 9.

References

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Appendix 2: List of bacterial species detected in catfish samples

Acidovorax sp.	*Comamonas testosteroni*	*Providencia* sp.
Acinetobacter sp.	*Comamonas*-like organism	*Pseudomonas* sp.
Acinetobacter baumannii	*Comamonas* sp.	*Pseudomonas aeruginosa*
Acinetobacter baylyi	*Cryptococcus magnus*	*Pseudomonas alcaligenes*
Acinetobacter guillouiae	*Curtobacterium* sp.	*Pseudomonas alcaliphila*
Acinetobacter junii	*Dickeya* sp.	*Pseudomonas anguilliseptica*-like
Acinetobacter radioresistens	*Edwardsiella ictaluri*	*Pseudomonas fluorescens* group
Acinetobacter tandoii	*Edwardsiella tarda*	*Pseudomonas gessardii*
Aeromonas sp.	*Elizabethkingia meningoseptica*	*Pseudomonas monteilii*
Aeromonas caviae	*Enterobacter cloacae*	*Pseudomonas oryzihabitans*
Aeromonas jandaei	*Enterococcus* sp.	*Pseudomonas otitidis*
Aeromonas hydrophila	*Flavobacterium* sp.	*Pseudomonas putida*
Aeromonas schubertii	*Flavobacterium lindanitolerans*	*Pseudomonas putida* group
Aeromonas trota	Fungal species	*Pseudomonas rhodesiae*
Aeromonas veronii	*Herbaspirillum huttiense*	*Pseudomonas stutzeri*
Aeromonas veronii biovar sobria	*Klebsiella oxytoca*	*Rhizobium radiobacter*
Arthrobacter sp.	*Kocuria* sp.	*Sarocladium* sp.
Arthrobacter gandavensis	*Lactococcus garvieae*	*Serratia fonticola*
Arthrobacter globiformis	*Lactococcus lactis*	*Shewanella* sp.
Arthrobacter polychromogenes	*Lactococcus raffinolactis*	*Shewanella putrefaciens*
Bacillus sp.	*Lysinibacillus fusiformis*	*Sphingomonas* sp.
Bacillus cereus group	*Morganella morganii*	*Staphylococcus* sp.
Bacillus licheniformis	*Microbacterium* sp.	*Staphylococcus epidermidis*
Bacillus marisflavi	*Microbacterium arborescens*	Unidentified yeast
Bacillus megaterium	*Micrococcus luteus*	Unidentified gram positive cocci
Bacillus mycoides	*Moraxella* sp.	Unidentified gram positive rod
Bacillus/Lactobacillis-like	*Moraxella osloensis*	Unidentified gram negative rod
Brevundimonas sp.	Non-haemolytic *E. coli*	Unidentified
Candida pelliculosa	*Paenibacillus illinoisensis*	Unidentified oxidase-positive gram negative rod
Chryseobacterium sp.	*Photobacterium damselae* ssp. *damselae*	Unidentified oxidase-negative gram negative rod
Citrobacter braakii	*Plesiomonas shigelloides*	*Vagococcus fluvialis*
Citrobacter freundii	*Proteus* sp.	*Vibrio cholerae* non01 non0139
Citrobacter freundii group	*Proteus vulgaris*	*Yokenella* sp.