THE EFFECT OF COOKING AND REFRIGERATION ON SURVIVAL OF APHANOMYCES ASTACI IN CULTURE AND IN CRAYFISH PRODUCT: DATA FOR IMPORT RISK ASSESSMENT

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Prevention of transmission of infectious disease under the SPS Agreement is one of the few reasons for which import of food species or food species product may be refused or subject to import restrictions. Crayfish plague has a 140 year history of destruction of susceptible native crayfish populations in Europe, can be carried by resistant species, and is listed as an "other significant disease" in the OIE International Aquatic Animal Health Code. Thus there is good reason to consider the application of controls on the import of crayfish into countries such as Australia where the disease is absent and native stocks are known to be susceptible. Although the disease is well known and the risks of importing potential live carrier crayfish clear, no valid information existed which would allow an assessment to be made on the risks associated with the import of crayfish product (e.g. frozen crayfish tail meat or cooked crayfish). On behalf of Biosecurity Australia, studies were carried out to determine whether and for how long the Oomycete fungal pathogen *Aphanomyces astaci*, which is the cause of crayfish plague could survive freezing or cooking temperatures.

Three approaches were taken: determination of susceptibility of mycelium, of zoospores and finally survival in infected crayfish. Mycelial colonies and spores were tested, using modified fungicide test protocols, to temperatures between +60°C and -20°C for periods between 5 minutes and 14 days. Effects of these exposures on survival and new growth were assessed. Finally, susceptible crayfish *Astacus leptodactylus* were infected with *A. astaci* and then cooked or frozen.

Effects of temperature on the growth and survival of *A. astaci* mycelium

Disk method

The results from the filter disk exposure method are presented in Figures 1 and 2. These graphs are plots of the mean results of the data derived from each time and temperature combination. *A. astaci* survived well at those intermediate temperatures, which are at, or near its normal growth range (0 to 10°C). The normal range growth presented in 3D graphical form obscures data from the opposite end of the time or temperature range.

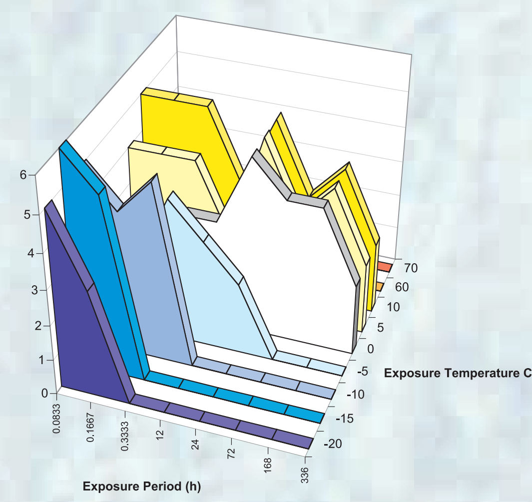

Figure 1: Mycelial growth of *A. astaci* after exposure to varying temperatures for different periods at 24h after return to normal temperatures (disk method).

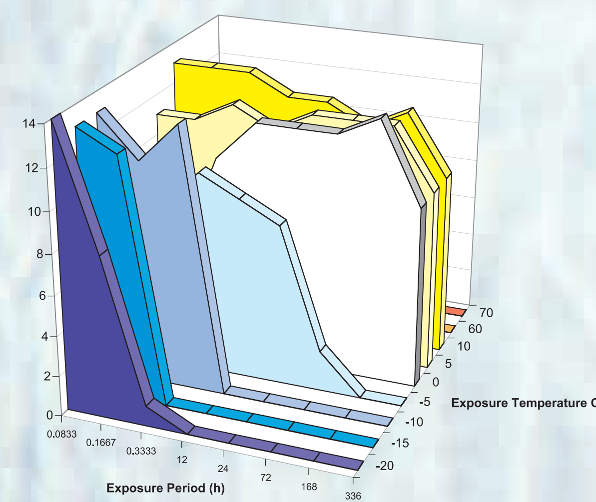

Figure 2: Mycelial growth of *A. astaci* after exposure to varying temperatures for different periods at 48 h after return to normal temperatures (disk method).

At 24 hours after the return of the fungal colony to its normal culture temperature of 15°C, no new growth had occurred from colonies exposed to temperatures of -20°C and -15°C for more than 10 minutes. At -10°C, exposure for more than 20 minutes prevented further growth at 24 hours as did exposure to -°5C for than 24 hours.

At the opposite end of the exposure temperature scale, no new growth had developed in 24 hours from colonies exposed for 5 minutes or more to +60°C or +70°C.

That *A. astaci* is able to recover from limited exposure to "abnormal temperatures" was demonstrated in colonies at 48 hours after return to normal 15°C culture temperature. Some limited survival was seen at 20 minutes exposure to -20°C and at -10°C, whilst at -5°C, growth was possible after 3 days exposure

The culture plates from the -20, -15 and -10°C 12 hour exposures were maintained for a further 4 days at 15°C (a total of 6 days post exposure to those temperatures) and no new growth of *A. astaci* was recorded.

At +60°C and +70°C no new growth occurred from colonies exposed to those temperatures for as little as 5 minutes

Agar plug method

Temperature sensitivity of *A. astaci* was tested by the agar plug method over the same time and temperature range as employed for the filter disk method. Results are presented in Figure 3. Again exposures to temperatures below -10°C prevented any further growth (after 48 hours incubation at normal temperature) for more than 10 minutes at -20°C, 20 minutes at -15°C and more than 12 hours at -10°C. This agreed with the result obtained after 24 hours normal incubation.

At 60°C for 5 minutes followed by 48 hours at normal incubation temperatures, A. astaci survived and some new growth was evident. No survival was noted for exposures of more than 5 minutes at 60°C, nor for up to 5 minutes exposure at 70°C.

Effects of temperature on the growth and survival of *A. astaci* spores

The results obtained were superficially fairly straightforward. When numbers of colonies were counted after 4 days incubation at 15°C (Figure 4), the numbers of colonies increased with length of exposure time to temperatures between 0°C and 15°C. A marked increase in numbers of new colonies occurred with temperature exposure times in excess of 24 (15°C) to 72 hrs (0°C). Below 0°C, exposures of up to 12h at -5°C and -10°C did not result in any reduction in the number of colonies produced on return to incubation at 15°C, but very few propagules survived 24h exposure to these temperatures. None survived to germinate when exposed for 72h.

Acknowledgement

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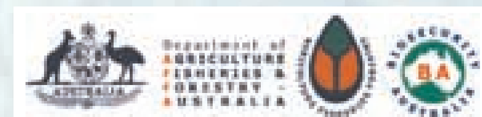
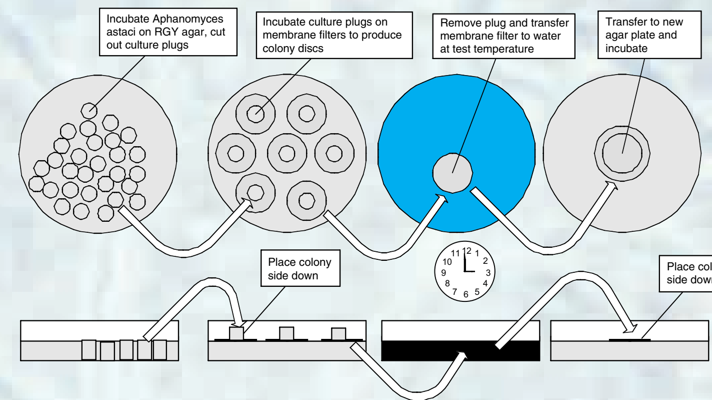
We are grateful for permission to publish these results.



Materials and Methods

The diagram to the right explains the "disc" method of testing in which plugs of actively growing colony are incubated on Polycar-bonate membrane filters to produce a "naked" mycelial colony that can be used for testing. The "plug" method uses colony plugs themselves and exposes these to test conditions. Measure of effect is the ability to produce new normal growth when returned to normal cultural conditions. Zoosporulation is induced in sterile distilled water, spores are exposed to test conditions in 6 well plates before transfer to normal conditions to determine effect on growth.



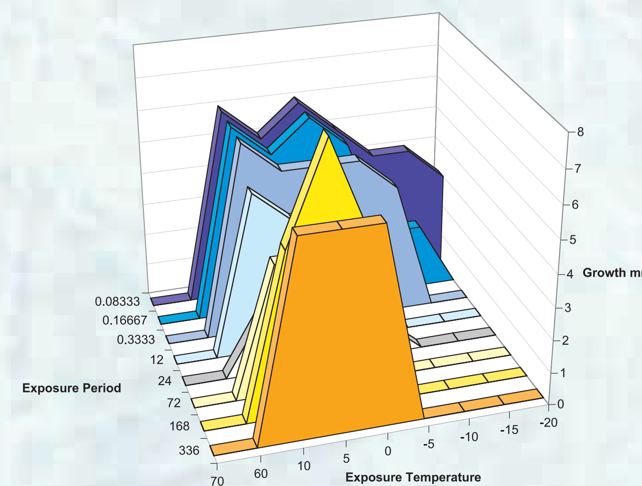


Figure 3: Mycelial growth of *A. astaci* after exposure to varying temperatures for different periods at 24h after return to normal temperatures (agar plug method).

At -15°C and -20°C a few spores survived to germinate when exposed for 5 or 10 minutes, none survived 20 minutes at these temperatures.

To confirm the results and give additional time for any spores slow to germinate and show visible growth after temperature shock, plates were incubated for an additional 3 to 4 days. During this time there was a slight increase in the number of colonies counted, but no change in the overall pattern. The pattern of results was almost identical when number of colonies was counted after 5 to 8 days post exposure incubation (Figure 5).

Effects of temperature on the growth and survival of *A. astaci* in crayfish

In the initial trial to determine percentage recovery of *A. astaci* from crayfish infected in the manner described above, recovery was achieved from 10 of 20 crayfish (50%). In practice this recovery percentage is considerably greater than that normally attained from crayfish in natural crayfish plague outbreaks and was deemed acceptable for the main study to proceed.

Recovery of *A. astaci* from the controls in the main trial was 40% (8 of 20). No recovery of A. astaci was successful for crayfish cooked at 100°C for one minute or from those frozen for 3h or 12h at -20°C.

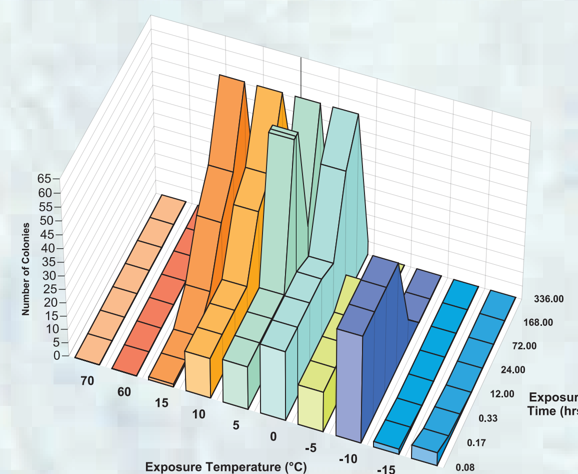


Figure 4: *Aphanomyces astaci* spore counts at 4 days after exposure to different temperatures

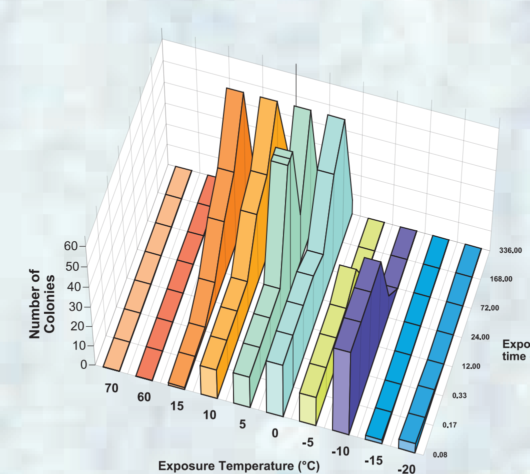


Figure 5: *Aphanomyces astaci* spore counts at 5-8 days after exposure to different temperatures

	Number of crayfish challenged	Number of infections recovered after temperature exposure	Percent Recovery
Control Crayfish	2 x 10	8	40
100°C, 1 minute	2 x 10	0	0
-20°C, 3h	2 x 10	0	0
-20°C, 12h	2 x 10	0	0

Discussion and Conclusions

For this discussion the term propagule is a useful one to employ, referring to all viable stages of *A. astaci* capable of propagating the organism including zoospores, aplanospores, encysted spores spore germlings and vegetative mycelium.

These results indicate that propagules of A. astaci have only limited ability to survive for periods of exposure to temperatures below 0°C for more than 24h and below -10°C for less than 20 minutes. At and above 60°C, no propagules survive even for 5 minutes. Therefore normal freezing or cooking procedures will ensure that no viable propagules will be present. In infected crayfish *A. astaci* will not survive normal cooking or freezing process times.

This simple interpretation of the results of this study in relation to commercial transfer hazards disguises a number of other scientifically interesting and difficult to interpret aspects of the study.

Oomycete zoospores react to fairly small physical shocks by encysting. At 15°C the spores will have received two such shocks in quick succession (5 to 20 minutes interval) when they were pipetted into the 6 well dishes and then out and onto the incubation plates. At a temperature at which the spores are active, the resulting poor survival should not be regarded as unexpected. When the transfer shocks were separated by 12h or more survival improved.

Although some suggestions can be made, the very interesting increase in numbers of surviving propagules which occurs after exposures of between 12 h and 72h at temperatures between 15°C and 0°C is much more difficult to explain. The result would seem to be linked to metabolic or physiological factors since the effect is delayed as exposure temperature is lower.

The short (5 to 10 minutes) period of survival at -15°C and -20°C reflects the difficulties of cooling a spore suspension down rapidly and it is unlikely that these results represent true temperature exposures for the full time. The survival of spores for up to 24h at -5°C and -10°C was however a little unexpected. Oomycetes are known to contain high molecular weight sugars in vacuoles in the cytoplasm. These have been suggested to offer possible limited cryoprotection to the cells for at least short periods. Certainly, *A. astaci* must normally be able to survive low water temperatures for periods in crayfish in Scandinavia beneath ice.

Most interestingly the length of exposure at which low temperatures (-5°C and -10°C) result in a sudden fall in survival of propagules is very much the same at that at which there is an increase in survival at 0°C and above. This gives added confidence in the results obtained both above and below 0°C.

The results suggest that some delayed germination or growth effect is occurring, the length of the lag period of which is affected by the temperature of exposure, increasing with reduction in temperature to 0°C. Below 0°C instead of increased numbers of colonies forming, all propagules are killed.

Published and unpublished observations on *A. astaci* indicate that under normal temperature conditions, germination will take place fairly rapidly (24h) and visible germlings can be observed on the agar surface in this time at 15°C. These observations refer to spores deposited directly onto RGY agar and incubated immediately at 15°C. In the present study, spores were held in distilled water at the test temperature. In both cases the spores were produced from mycelial culture that had been washed several times in sterile distilled water before incubating in distilled water to induce zoosporulation. Availability of nutrients in the spore containing water was therefore low.

Encysted spores of Oomycetes can germinate under low nutrient conditions to produce germlings or they may excyst directly to produce another motile zoospore. Such germlings are short lengths of narrow vegetative mycelium which, as reported, can then produce a small spherical terminal cyst structure capable of releasing a single zoospore. This has been termed "repeated emergence". Whilst there is no specific report of repeated emergence occurring in *A. astaci*, germlings are produced in distilled water and these may become septate. The possibility exists that such septate germlings may be fragmented during transfer and thus act as more than one propagule giving a possible explanation for the increase in colonies noted after 24 to 72h exposure at 15°C down to 0°C.

This interpretation leads to the suggestion that the colonies appearing after short exposures represent largely zoospores which have not encysted and have remained motile and able to germinate rapidly. Those growing after the longer exposures could thus represent encysted spores and germlings. Few zoospores (or encysted spores or germlings) survived exposures to -5°C or -10°C for 12h and none for exposures of 24h.

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