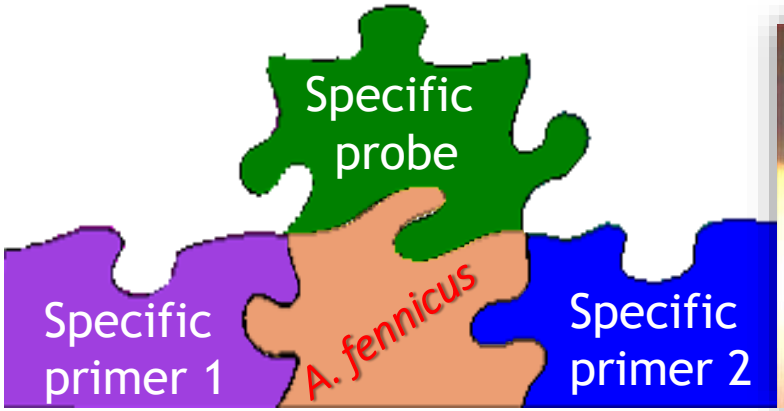
# Update on the development of a qPCR assay that discriminate *A. astaci* from *A. fennicus*





***Aphanomyces astaci* specific qPCR ?**


Trude Vrålstad

Head of Fish Health Research Group


10th AW of the NRL for Crustacean Diseases – May 29th 2019

Veterinærinstituttet
Norwegian Veterinary Institute

# Before 2006 ish: Problems to diagnose the crayfish plague

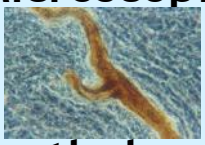
## The ancient toolbox for crayfish plague diagnostics

Indication

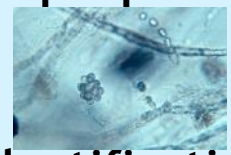
Field diagnosis
+ Disease observations

Gross pathology

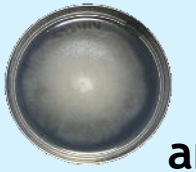
Microscopic pathology



Microscopic presumptive identification



Isolation in pure culture
Verification of identity and/or virulence



Confirmation

Very low success rate in most diagnostic laboratories……

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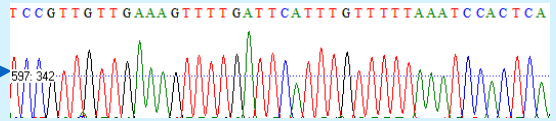
# From 2006 ish: Developing new tools to diagnose the crayfish plague

## The molecular toolbox for crayfish plague diagnostics (OiE recommended)

Specific PCR – direct molecular detection from clinic samples

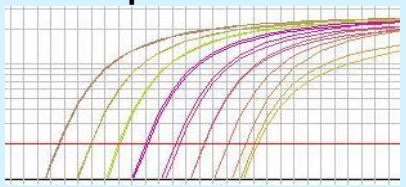
Oidtmann et al, 2006

DNA sequencing




Identity verification

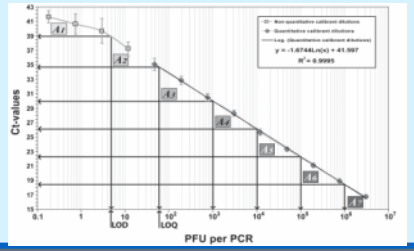
Specific



real-time PCR

Vrålstad et al, 2009

Quantification of *A. astaci*




Confirmation

High success rate in most diagnostic laboratories……

# From 2006 ish: Developing new tools to diagnose the crayfish plague

## The molecular toolbox for crayfish plague diagnostics (OiE recommended)

Oidtmann et al, 2006

Vrålstad et al, 2009

*Table 5.2. Methods for targeted surveillance in highly susceptible crayfish species to declare freedom from infection with A. astaci*

| Method | Screening method | Confirmatory method |
|---|---|---|
| Inspection for gross signs and mortality | a | c |
| Microscopic signs (wet mounts) | c | c |
| Isolation and culture | c | b |
| Histopathology | d | d |
| PCR | a | b, possibly a[1] |
| qPCR | a | b, possibly a[1] |
| Sequencing of PCR products | n/a | a |
| Transmission EM | n/a | n/a |
| Antibody-based assays | n/a | n/a |
| *In situ* DNA probes | n/a | n/a |

PCR = polymerase chain reaction; qPCR = quantitative PCR; EM = electron microscopy; n/a = not applicable or not available;

[1] = see definitions of confirmed case in Section 7.1

High success rate in most diagnostic laboratories……

# *Aphanomyces fennicus* sp.nov

A new species very closely related to *A. astaci* (Viljamaa-Dirks & Heinikainen, 2019)

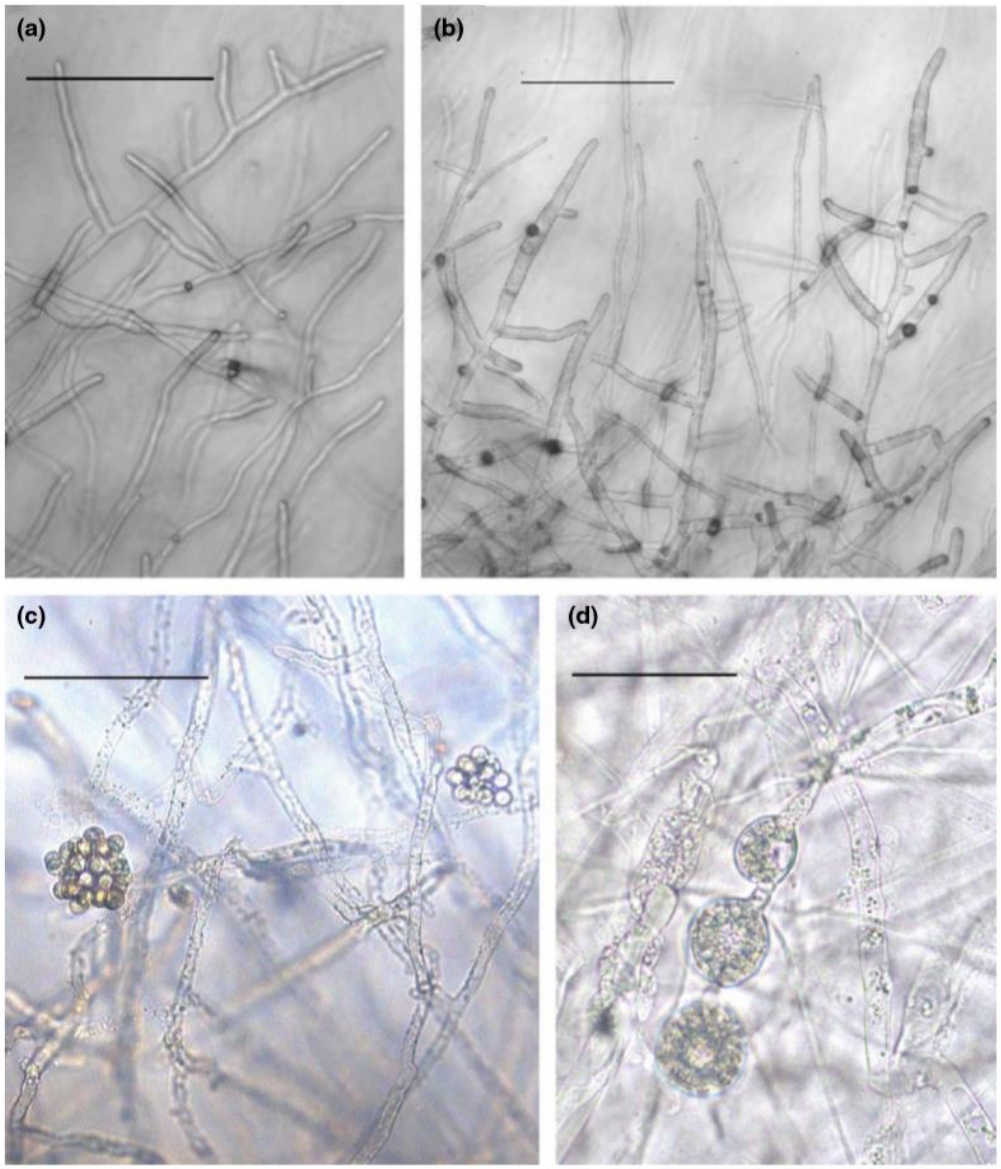
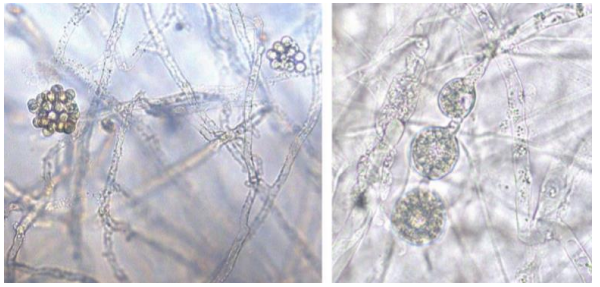


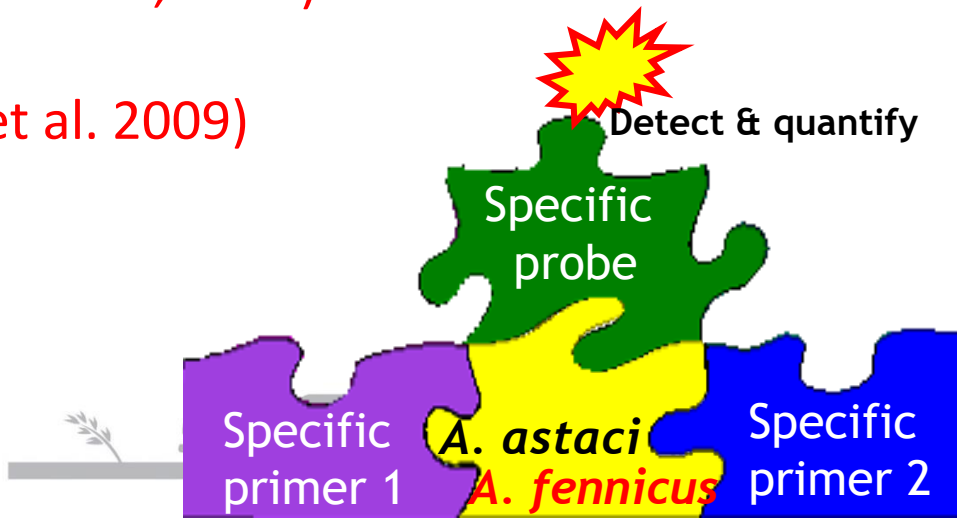

**FIGURE 1** Morphological features of the isolates of novel *Aphanomyces fennicus* sp. nov.: (a) hyphae, (b) hyphal enlargements, (c) spore balls, (d) chlamydospore-like structures. (a and c) are similar to *Aphanomyces astaci*, (b) is rarely, and (d) almost never seen in *A. astaci* cultures. Scale bar: (a and b) 200 µm, (c) 100 µm, (d) 50 µm [Colour figure can be viewed at wileyonlinelibrary.com]

# Facts and challenges



- *Aphanomyces fennicus* sp. nov.
- Isolated from Noble crayfish in Finland
- Distinguished from *A. astaci* morphologically
- A-virulent, no mortality observed
- Slighly different ITS-sequence
- Unique RAPD-genotype & microsat-genotype
- Not distinguished from *A. astaci* by the current PCR/qPCRs
  - ITS – conventional PCR (Oidtman et al, 2006)
  - ITS- qPCR (Vrålstad et al. 2009)
  - Chitinase qPCR (Hochwimmer et al. 2009)

*Viljamaa-Dirks & Heinikainen, 2019*

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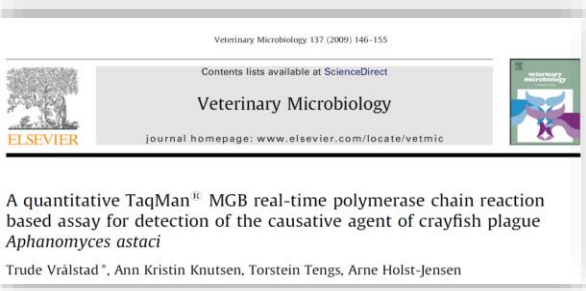
# ITS-qPCR used for diagnostics and eDNA monitoring



Veterinary Microbiology 137 (2009) 146–155

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*

Trude Vrålstad*, Ann Kristin Knutsen, Torstein Tengs, Arne Holst-Jensen

50 YEARS WITH IMPACT

Journal of Applied Ecology

*Journal of Applied Ecology* 2014, **51**, 544–553 doi: 10.1111/1365-2664.12218

**Detection of crayfish plague spores in large freshwater systems**

David A. Strand[1,2], Japo Jussila[3], Stein I. Johnsen[4], Satu Viljamaa-Dirks[5], Lennart Edsman[6], Jannicke Wiik-Nielsen[1], Hildegunn Viljugrein[1], Frederik Engdahl[6] and Trude Vrålstad[1,2]*

Received: 29 November 2018 | Accepted: 10 March 2019

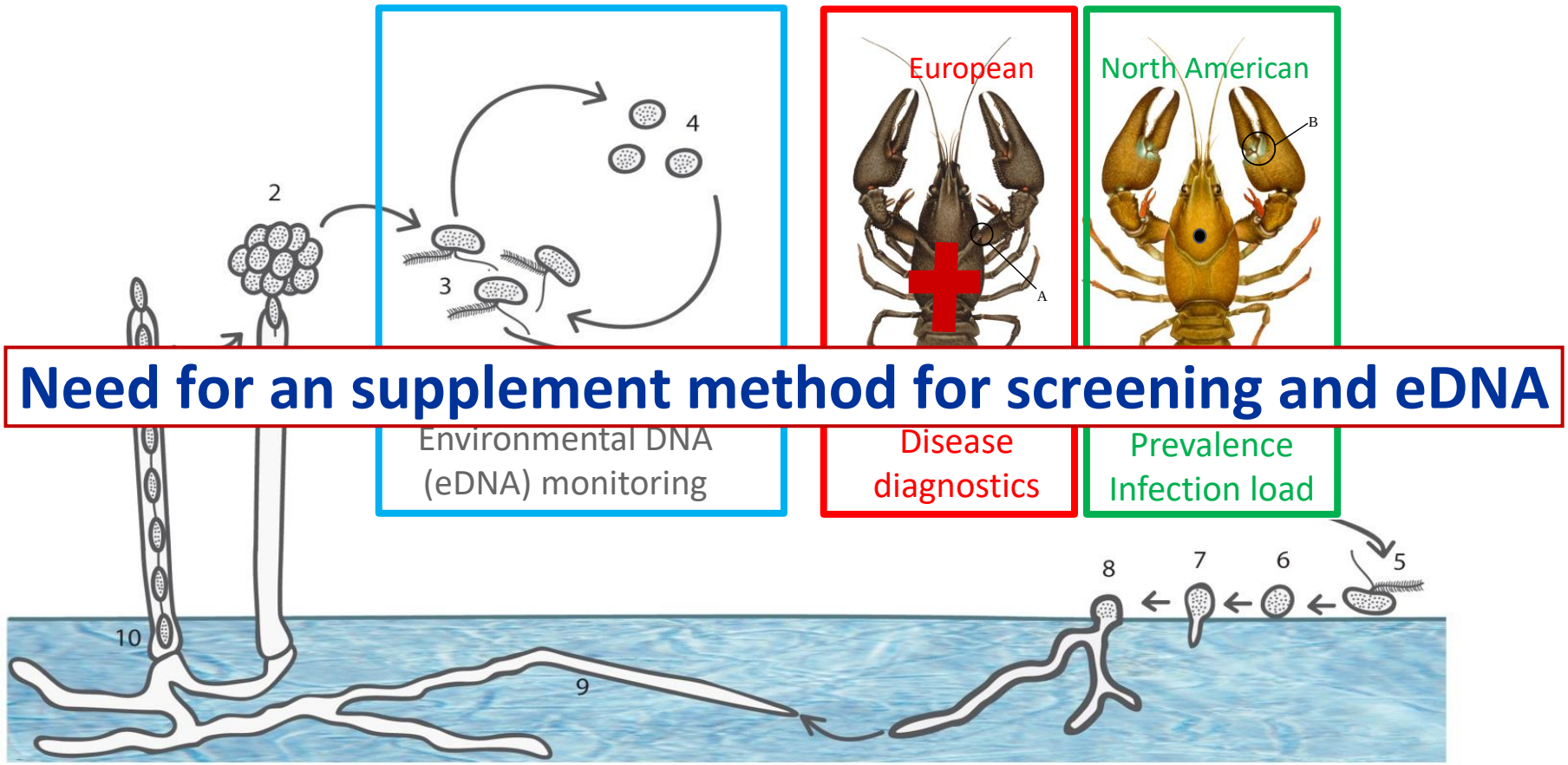
DOI: 10.1111/1365-2664.13404

RESEARCH ARTICLE

Journal of Applied Ecology

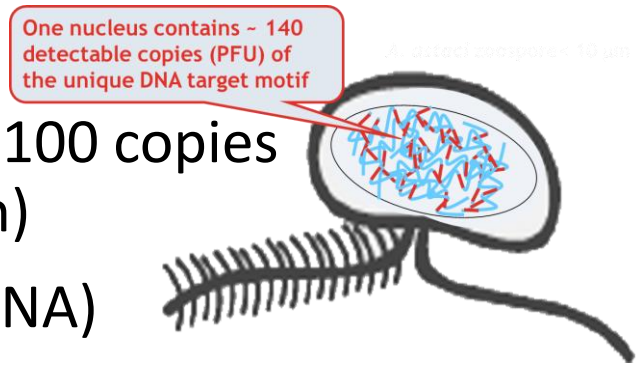
**Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction**

David A. Strand[1,2] | Stein Ivar Johnsen[3] | Johannes C. Rusch[1,4] | Sune Agersnap[5,6] | William Brenner Larsen[5] | Steen Wilhelm Knudsen[5] | Peter Rask Møller[5] | Trude Vrålstad[1]

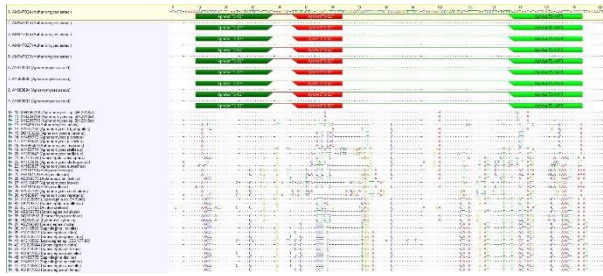



**Need for an supplement method for screening and eDNA**

# Motivation for ITS nrDNA as target region for a new qPCR




- Each nucleus in cells of *A. astaci* contains >100 copies (high sensitivity and likelihood of detection)
- Bar-code region (along with Cox 1 & 2 mtDNA)
- High number of (relevant) oomycete taxa sequenced
- Easy to play alternative assays within the previous region of choice (= most variable part of the ITS-regions for Saprolegniaceae)

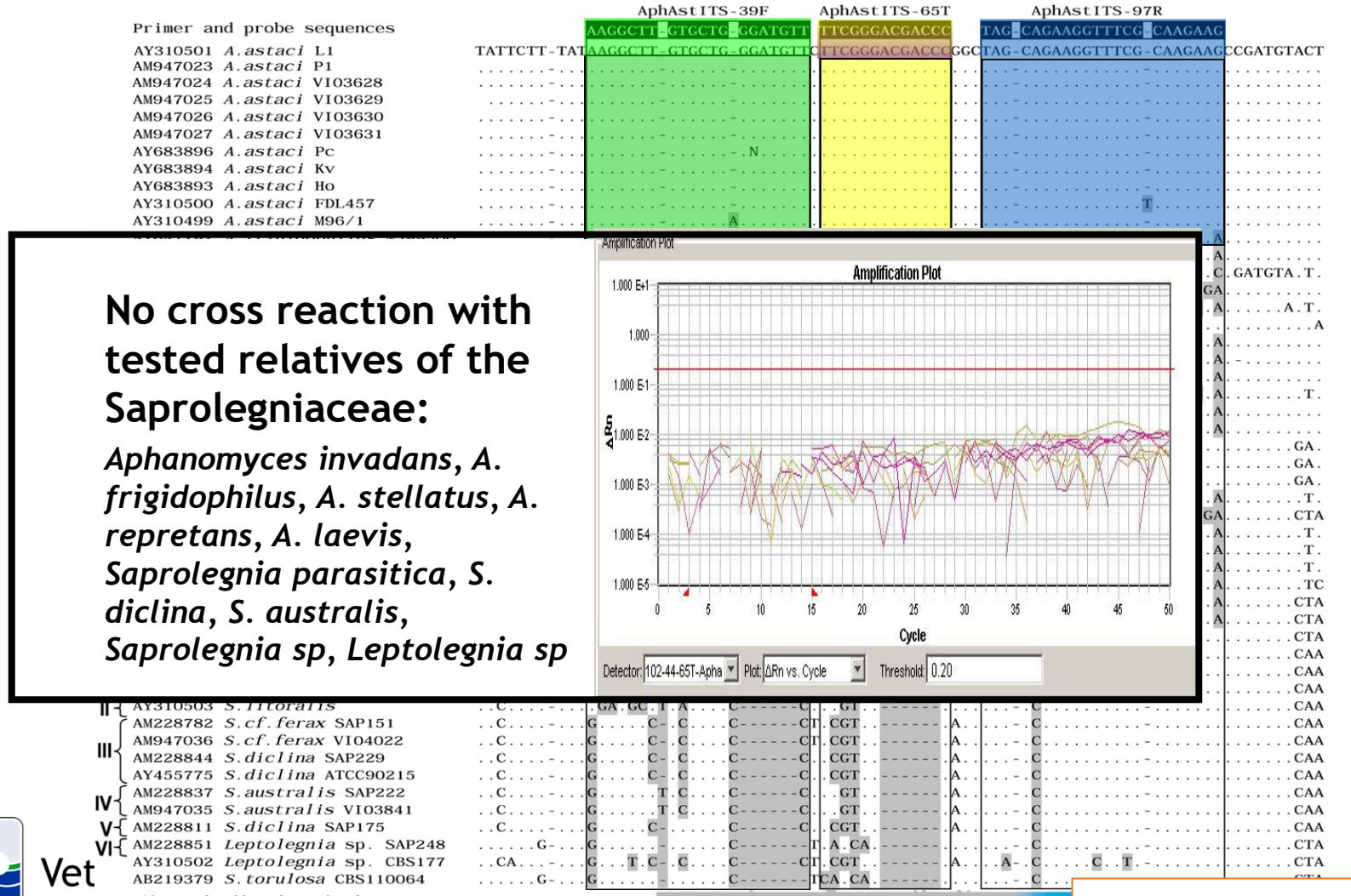


- Low-cost alternative

- COX2 (and other promising regions) should also be considered



# TaqMan® MGB Real-time PCR assay for detection of *A. astaci*




**No cross reaction with tested relatives of the Saprolegniaceae:**

***Aphanomyces invadans*, *A. frigidophilus*, *A. stellatus*, *A. repretans*, *A. laevis*, *Saprolegnia parasitica*, *S. diclina*, *S. australis*, *Saprolegnia sp*, *Leptolegnia sp***

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Vrålstad et al, 2009

# *A. astaci* and *A. fennicus* - identical probe region

**TABLE 2** The ITS segments used for the real-time PCR (Vrålstad et al., 2009) probe and primers and for the diagnostic PCR protocols (Oidtmann et al., 2006, 2004) in comparison with the gene segments of the *Aphanomyces fennicus* sp. nov. isolates M6/1, M6/2 and M7/3

| | Primer AphAstITS-39F | | | | | | | | | Probe AphAstITS-60T | | | | | Primer AphAstITS-97R | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aphanomyces astaci* | ttat | AAG | GCT | TGT | GCT | GGG | ATG | TT | c | TTC | GGG | ACG | ACC | C | ggc | TA | GCA | GAA | GGT | TTC | GCA | AGA | AG | ccg |
| M6/1 | .... | ... | ... | ... | ... | ... | .C. | .. | . | ... | ... | ... | ... | . | ... | .. | ... | ... | ... | ... | ... | ... | .A | ... |
| M6/2 | .... | ... | ... | ... | ... | ... | .A. | .. | . | ... | ... | ... | ... | . | ... | .. | ... | ... | ... | ... | ... | ... | .A | ... |
| M7/3 | .... | ... | ... | ... | ... | ... | .A. | .. | . | ... | ... | ... | ... | . | ... | .. | ... | ... | ... | ... | ... | ... | .A | ... |

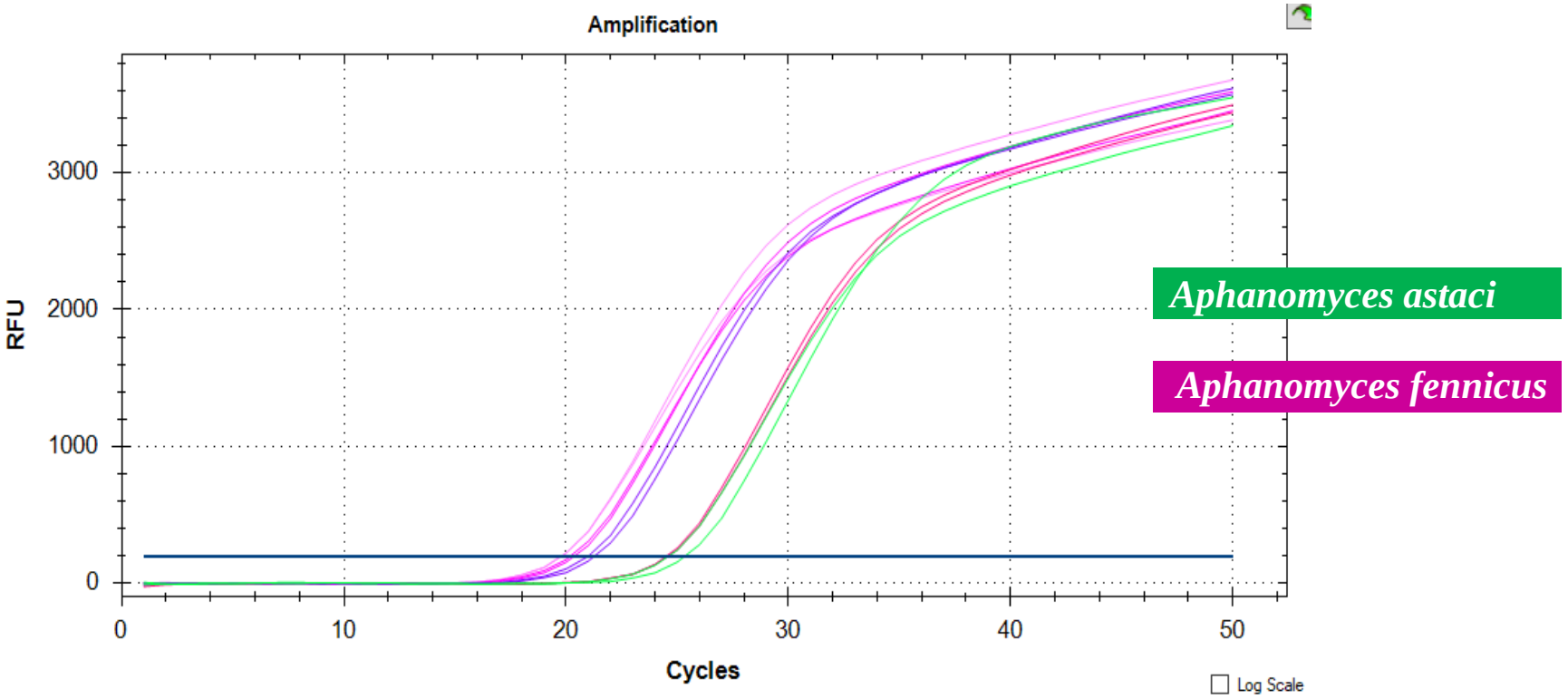
| | Primer BO42 | | | | | | | | Primer BO525 | | | | | | | | Primer BO640 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. astaci* | GCT | TGT | GCT | GAG | GAT | GTT | CTT | // | AAG | AAG | GCT | AAA | TTG | CGG | TA | // | CAG | AAT | GCG | GAG | TCG | GAT-AG | AG |
| M6/1 | ... | ... | ... | .-. | ..C | ... | ... | // | .G. | ... | .A. | G.. | ... | ... | ... | // | ... | ... | ... | ... | .T. | ... | ... |
| M6/2 | ... | ... | ... | .-. | ..A | ... | ... | // | .G. | ... | .A. | G.. | ... | ... | ... | // | ... | ... | ... | ... | .T. | ... | ... |
| M7/3 | ... | ... | ... | .-. | ..A | ... | ... | // | .G. | ... | .A. | G.. | ... | ... | ... | // | ... | ... | ..R | ... | .T. | ... | ... |

Note. Differences are highlighted in bold. R: G or A (consistent polymorphism analysed in two separate sequences).

*Viljamaa-Dirks & Heinikainen, 2019*

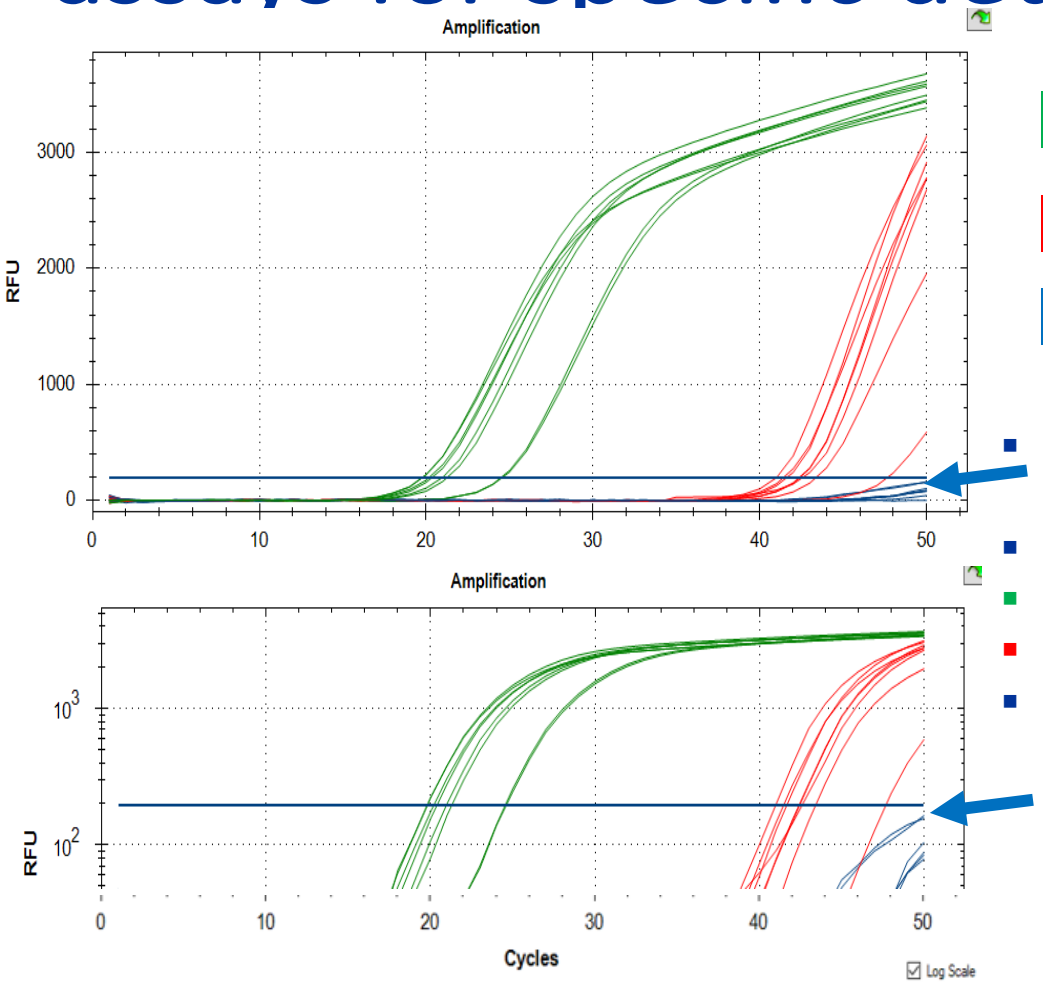
WILEY Journal of Fish Diseases






- Amplification of *A. fennicus* (M6/1, M6/2, M7/3, Matti 17/3: different shades of purple) and *A. astaci* (VIO3628: green) using Vrålstad et al. (2009) (with 62 °C)
- No difference in amplification efficiency – clear false positive

# Test of the old and two new candidate assays for specific detection of *A. astaci*




**Green - *A. fennicus "old" assay***

**Red - *A. fennicus "New I" assay***

**Blue - *A. fennicus "New II" assay***

- Comparing of the "Old", "New I" and "New 2" on genomic DNA from four *A. fennicus* strains
- Results are shown with and without log scale.
- "Old" assay yields clear cross amplification
- "New I" amplifies the strains after ct 40.
- "New II" assay: no cross-reaction observed
  - promising for further tests

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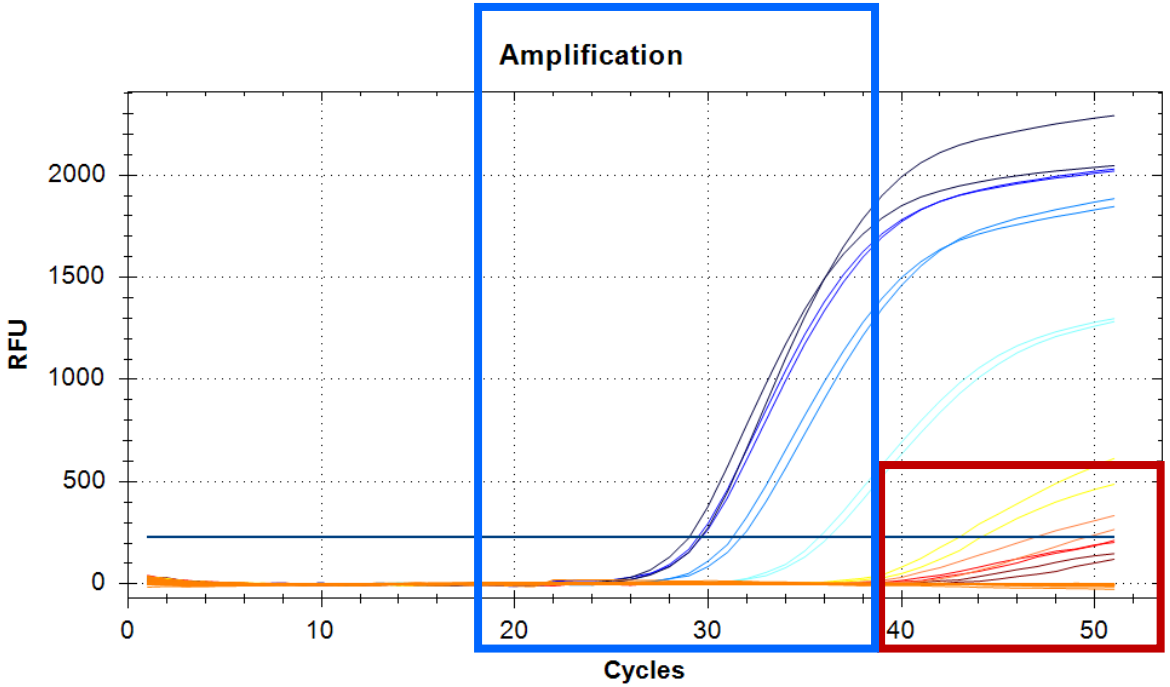
# Optimal temperature for the “NEW II” 62.6 °C for discrimination of *A. fennicus*

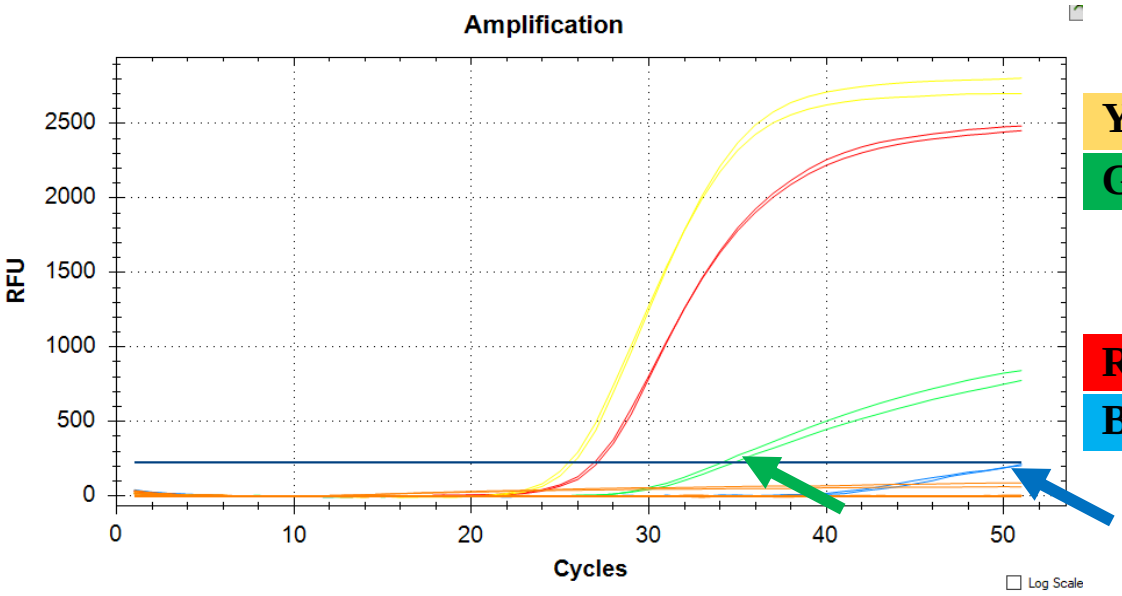



Dark red – 63 °C
Red 62,6 °C
Orange 61,7 °C
Yellow 60,1 °C

Light Blue 58,2 °C
Blue 56,6 °C
Dark blue 55,5 °C
Black 55 °C

- Gradient qPCR (55-63 °C) using the “New II” assay.
- *A. fennicus* not amplified at 60 °C and above while amply at 58 °C and below.
- Only tested for Taqman Environmental mastermix (EMM).

# "NEW II" test for *A. fennicus* and *A. astaci* - Choice of master mix matters




**Yellow - *A. astaci* with iTaq**

**Green – *A. fennicus* iTaq**

**Red - *A. astaci* with EMM**

**Blue – *A. fennicus* EMM**


- Gradient qPCR (at 62.6 °C) using using both mastermixes
- *A. fennicus* is amplified (weak amplification) using iTaq but not EMM

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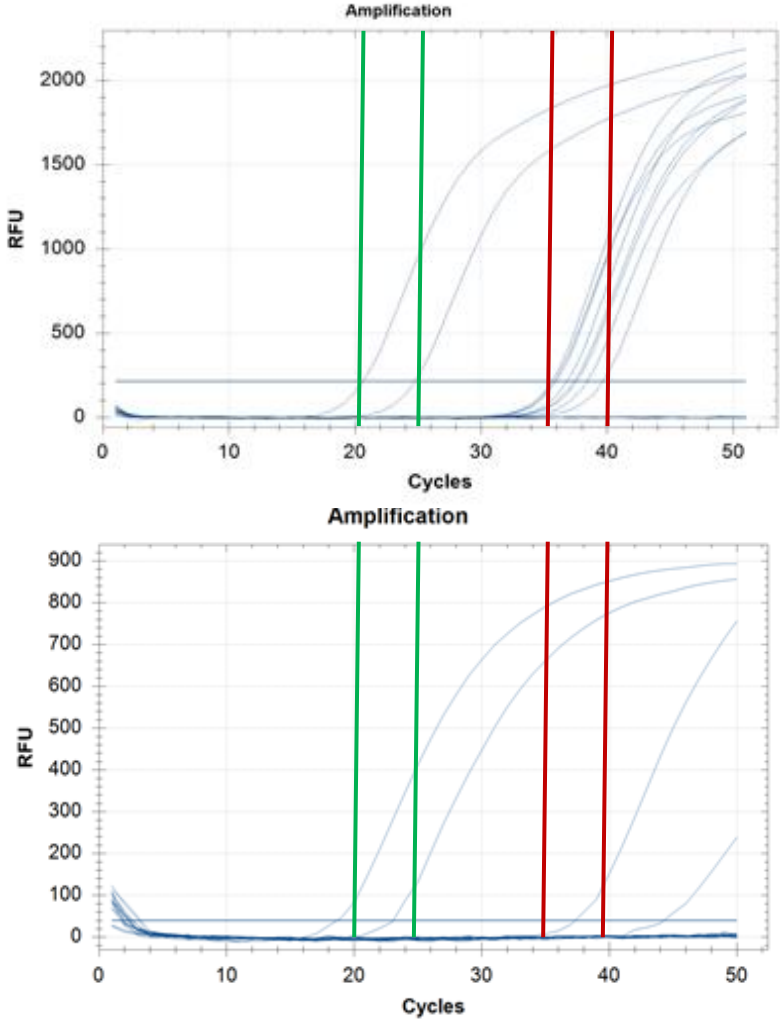
# Test in Finland: Better sensitivity with the “old” assay?




- Sensitivity old and “new 2” compared for pure culture DNA samples and infected tissue samples
- Same performance for pure culture
- Loose most of the low level positives in tissue samples (iTaq and EMM)
- Low level positives not possible to sequence, cultures almost never obtained.
- Can this be A. fennicus?

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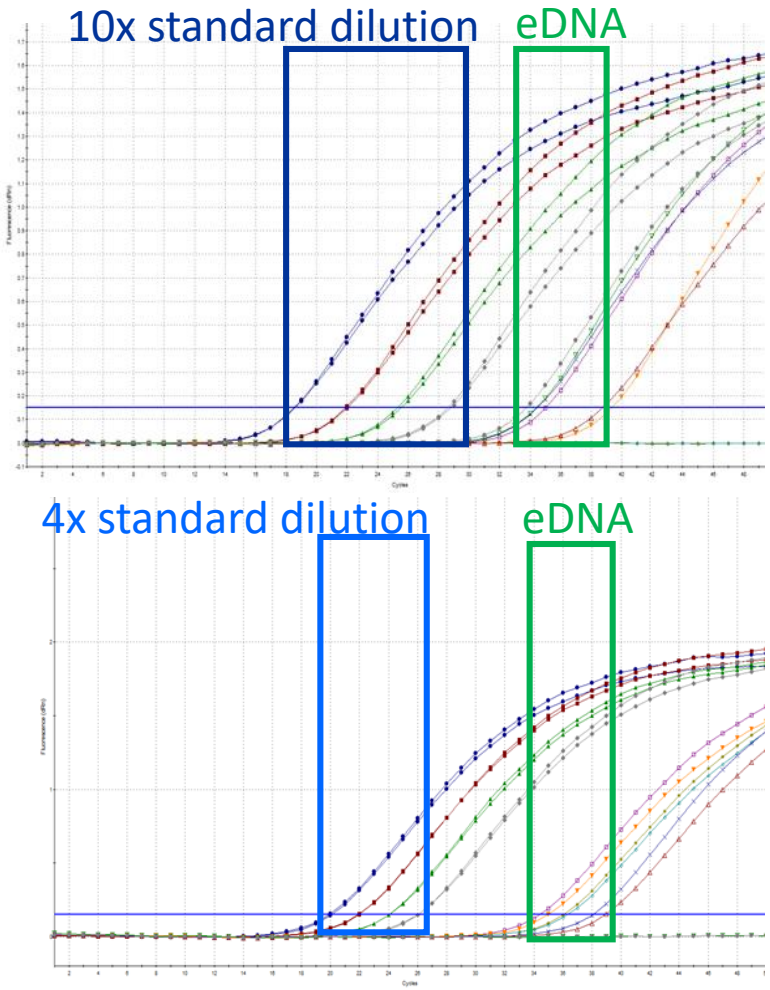
# Test in Norway: Apparently same performance on environmental samples




- Sensitivity old and “new 2” compared for environmental samples (confirmed *A. astaci*)
- Comparable results for eDNA samples
- Ct in the range from 33-39

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# Summary


- Promising results – although problematic that different mastermixes yields different specificity
- Validation work remains – tested against far less species than the “old” assay
- For now best to use as a second verification for positive samples with the “old” assay
  - Broad specificity better tested for the old than the new
- Publication remains…
- Interested to test or contribute? Send us a request – we share!

# Acknowledgements


- TARGET (NFR-293407)
- Norwegian Veterinary Institute
- Finnish Food Safety EVIRA, Finland
- Co-authors and collaborators
  - David Strand, Elin Rolén, NVI
  - Satu Viljamaa-Dirks and Sirpa Heinikainen, EVIRA Finland