A longitudinal study of amoebic gill disease on a marine Atlantic salmon, *Salmo salar* L., farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*.

J. Downes[1,2*], K. Henshilwood[1], E. M. Collins[1], Abdon Ryan[3], I. O'Connor, H. Rodger, E. MacCarthy[2], N. M. Ruane[1]

Jamie Downes
Jamie.downes@marine.ie

Marine Institute
Foras na Mara

GMIT
GALWAY-MAYO INSTITUTE OF TECHNOLOGY
INSTITIÚID TEICNEOLAÍOCHTA NA GAILLIMHE-MAIGH EO

MARINE AND FRESHWATER RESEARCH CENTRE
LÁRIONAD TAIGHDE MARA AGUS FIORUISCE

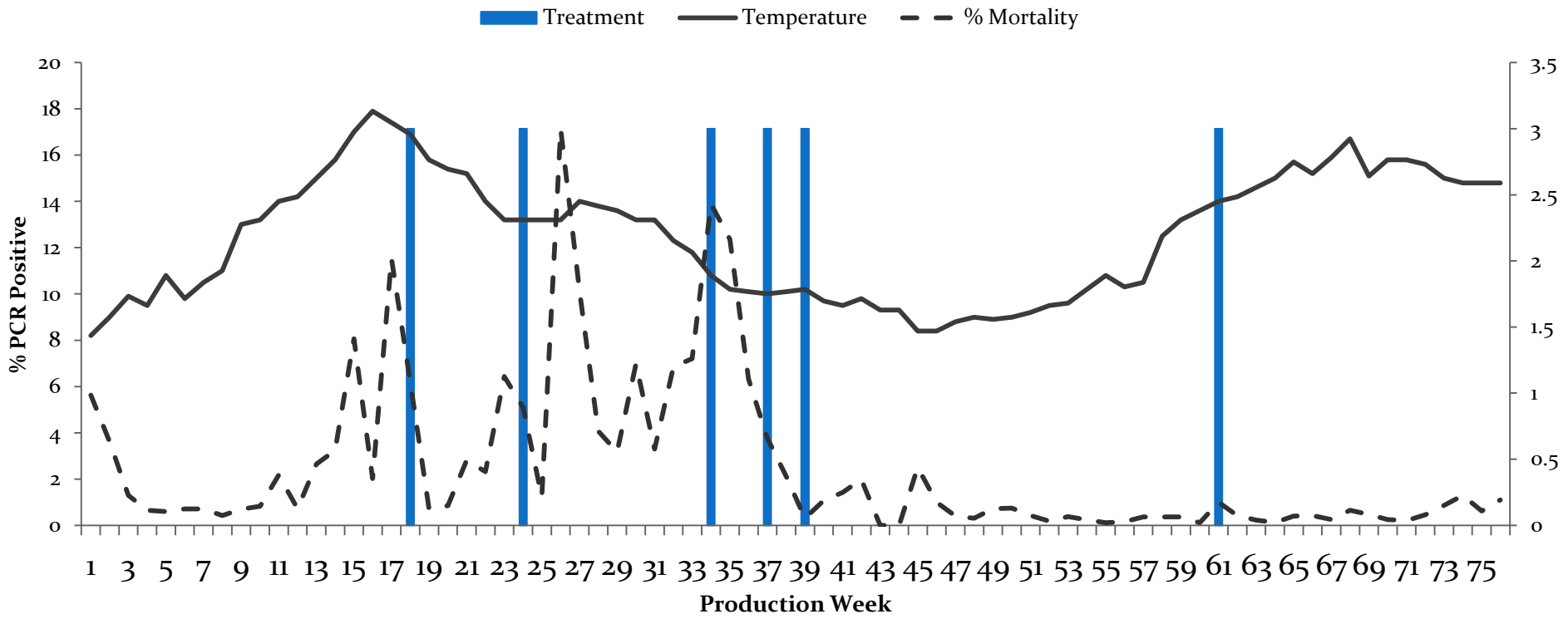
Vet Aqua INTERNATIONAL

A longitudinal study of amoebic gill disease on a marine Atlantic salmon, *Salmo salar* L., farm utilising a real-time PCR assay for the detection of *Neoparamoeba perurans*.

- Site in the South West of Ireland was selected to be included in the Longitudinal study.
- Sampling commenced 4 weeks post transfer (May 2013) up until week 76 (September 2014)
- Samples were collected every 2-3 weeks
- Gill scoring, histology, PCR
- TaqMan PCR was developed and validated for use in screening for *N. perurans*

Longitudinal study

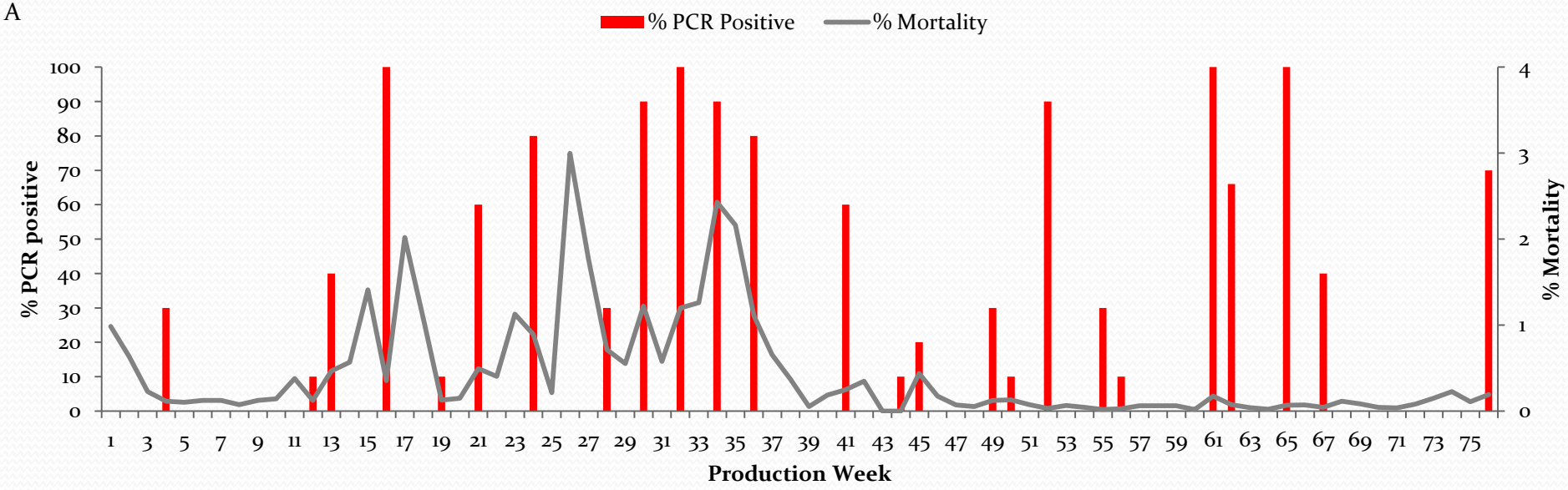
- 3 observed peaks in mortality due to AGD, additionally large peak in mortality due to *P. noctiluca*
  - *2 full treatments, 4 partial*
- Following drop in temp and fish above 1kg mortality returned to background levels



Molecular screening

- PCR methods appears to be sensitive enough to pick up infection 2/3 weeks prior to gross assessment and track proliferation of amoeba post treatment
- Samples collected 4 days post treatment saw immediate reduction in positive fish, further increase 3 weeks observed post treatment
- Mortality was reduced to background levels, still having detections and gill pathology, important to take all information into account.

A



Evaluation of non-destructive molecular diagnostics for the detection of *N. perurans*

Jamie Downes
Marine Institute, Ireland
Jamie.downes@marine.ie

CSIRO Staff

- Mat Cook
- Richard Taylor
- Megan Rigby
- Ben Maynard

Marine Institute
Foras na Mara

GMIT
GALWAY-MAYO INSTITUTE OF TECHNOLOGY
INSTITIÚID TEICNEOLAÍOCHTA NA GAILLIMHE-MAIGH EO

MARINE AND FRESHWATER RESEARCH CENTRE
LÁRIONAD TAIGHDE MARA AGUS FIORUISCE

CSIRO

Evaluation of non-destructive molecular diagnostics for the detection of *P. perurans*

- Much effort has focused on molecular diagnostics with limited use in screening
    - Norway and recently Ireland
- No. of different assays and sampling methods in use
- Project would allow the evaluation of suitability of each assay and non-destructive sampling methodology
- Collaboration between CSIRO, Australia, Marine Institute, Ireland and GMIT, Ireland
- Preliminary results from the project

Aims

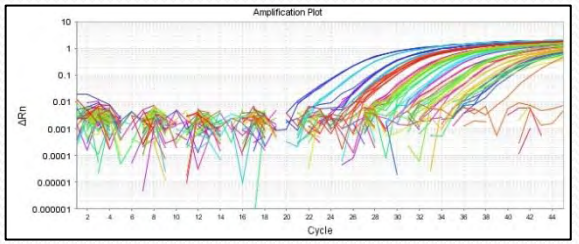
1. Assess most appropriate non-destructive sampling methodology
   - Swabs/filament clip
2. Determination of the most appropriate molecular assay with regard to sensitivity and specificity.
3. Comparison of diagnostic/screening methods
   - Gill score + Molecular
4. Correlation of non-destructive molecular diagnostics to the clinical gill score
5. Standardised methodology for field sampling and molecular diagnosis of AGD

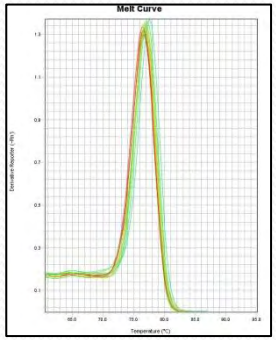
Sampling

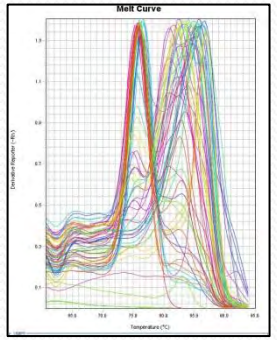
- 30 fish over the full range of gill score were sampled.
- Individual gill scores were recorded for each fish
- Two methods for non-destructive molecular sampling were examined.
  - Filament biopsy - ethanol
  - Gill swab (Isohelix swabs) - ethanol
    - Front and back of second gill arch were swabbed
- Additionally cultures from 3 countries were included for testing (Australia, Ireland, Norway)
- Known quantities of amoebae cells were also assessed

PCR assays included

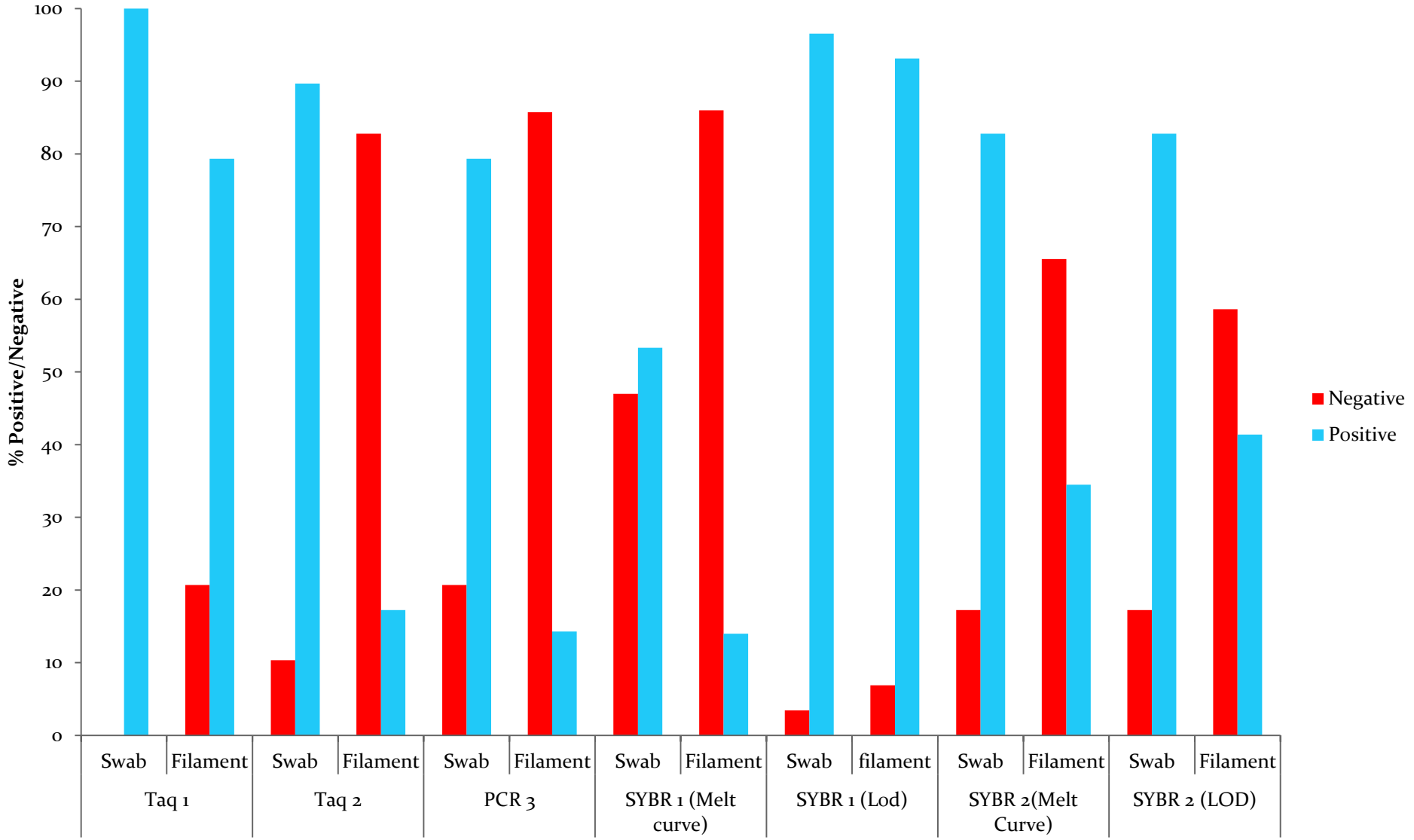
- 5 different assays available for use for trial
  - 2 TaqMan assay
  - PCR 3 (Partial analysis)
  - SYBR green 1
  - SYBR green 2 (Nested)







Swab/Filament Results



Swab/Filament

- Potential reasons for difference
  - Surface area sampled
    - Filament only covers small area 25 +/- 2 mg
    - Large surface area is covered by swab
    - Potentially more amoeba in the mucous
  - Competition
    - Assays may not be specific enough, may be competing DNA
  - Inhibition

Determination of the most appropriate molecular assay with regard to specificity and sensitivity.

- The percentage of samples positive for the presence of *N. perurans* was assessed for each assay
- Assay Taq 1 was recorded as having 100% positive with the gill swabs with 23% difference between swabs and filaments
- Taq 2 preformed relatively well with 90% but performance dropped dramatically when testing the filaments
- Both SYBR green assays required adjustment of results due to unspecific amplification following melt curve analysis

	Taq 1		Taq 2		PCR 3		SYBR 1(Melt curve)		SYBR 1(LOD)		SYBR 2(Melt Curve)		SYBR 2 (LOD)	
	Swab	Filament	Swab	Filament	Swab	Filament	Swab	Filament	Swab	filament	Swab	Filament	Swab	Filament
Negative	0	21	10	83	21	86	47	86	3	7	17	66	17	59
Positive	100	79	90	17	79	14	53	14	97	93	83	34	83	41

Determination of the most appropriate molecular assay with regard to specificity and sensitivity.

	Known No. Of Cells					
	v100	**v50**	**v25**	**v10**	**v5**	**v1**
Taq 1		30.04	31.26	32.37	32.98	35.10
Taq 2		30.43	31.29	32.35	33.32	35.51
SYBR 1	28.06		30.89	31.24		36.43
SYBR 2	22.97		26.05	27.27		39.39

- Both Taq and SYBR assay were used to analysis known No. of cells
- Each assay preformed well with known cells
- Both TaqMan assays were very similar and preformed best on single cell
- Further work to be completed.

- Cultures from 3 countries (Australia, Ireland and Norway) were analysised
  - All assays performed equally well

Correlation of non-destructive molecular diagnostics to the clinical gill score

Correlation of Gill score and Molecular Results				
	Swab		Filament	
Taq 1	-0.722	P<0.01	-0.608	P<0.01
Taq 2	-0.629	P<0.01	-0.545	P<0.01
PCR 3	-0.163	P>0.05	-0.032	P>0.05
SYBR 1 (Melt Curve)	-0.665	P<0.01	-0.446	P<0.05
SYBR 1 (LOD)	-0.740	P<0.01	-0.521	P<0.01
SYBR 2 (Melt Curve)	-0.717	P<0.01	-0.632	P<0.01
SYBR 2 (LOD)	-0.717	P<0.01	-0.554	P<0.01

- Correlation is greater with the swabs when compared with filament in all assays.
- Correlation between the majority of the assays was relatively similar for these samples
- Correlation is variable

Correlation of non-destructive molecular diagnostics to the clinical gill score

- Correlation investigated on three separate sets of samples, 30 from trial, samples from infection trial, additional 42 samples taken previous sampling occasion were tested
- Correlation is significant
- However, can and does fluctuate due to several potential variables in gill scoring and sampling
  - Subjective gill scoring
  - Variations in area swabbed and amoeba load
  - And response of fish to amoeba
  - Brings up question of Resistance or Resilience

Correlation of Gill score and Ct values			
Taq 1	**Additional**	-0.828	P<0.01
	Current	-0.722	P<0.01
	Infection	-0.938	P<0.01

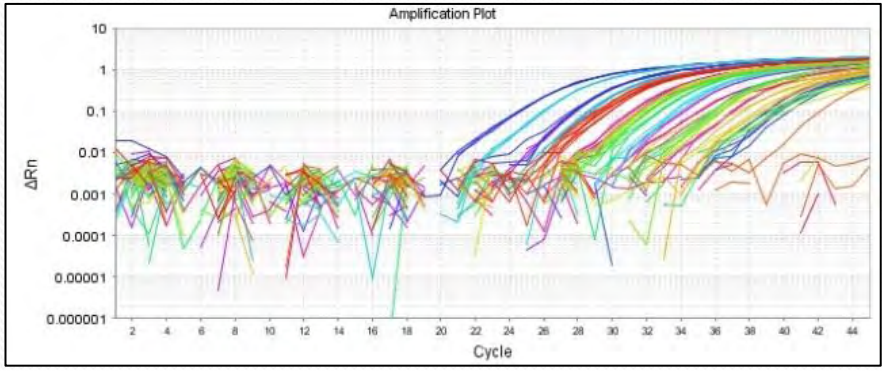
Correlation of non-destructive molecular diagnostics to the clinical gill score

Gill score	TaqMan 1	TaqMan 2	Sybr 1 Melt	Sybr 1 (LOD)	Nested (Melt Curve)	Nested (LOD)
0	A	A	A	A	A	A
1	AB	A	AB	A	A	A
2	AB	A	A	A	A	A
3	BC	A	AB	A	AB	AB
4	C	B	BC	B	B	B
5	C	B	C	B	B	B

- Gill scores that do not share a letter are significantly different
- Two to three groupings of Gill scores are observed in Tukey's post hoc test

Comparison of screening methods

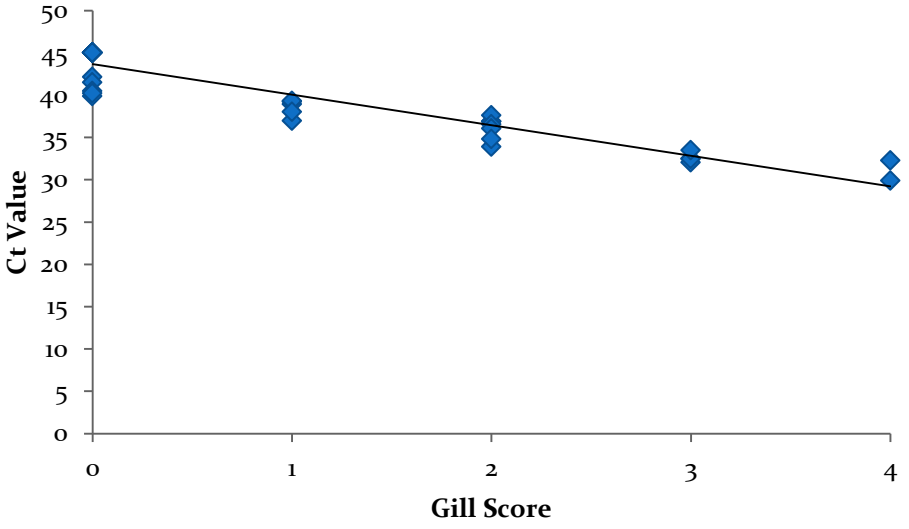
- Comparison of screening methods in order to determine the most suitable method for the ongoing monitoring of amoebic gill disease.
  - Gill score, Histology and Molecular
- 40 Fish sampled from infection trial with 1000 cells/L
  - 1, 2, 3, 8, 15, 21 days post infection



Comparison of screening methods

- Observed gill and histology scores appeared 1 week P.I
- Amoebae first observed in histology 2 weeks P.I
- Molecular diagnostics picked up amoebae 48hrs post infection
- Significant correlation between each of the methods P>0.01

	Gill score	PCR
PCR	-0.938	
Histo scores	0.849	-0.836



Standardised methodology for field sampling and molecular diagnosis of AGD

- Further work will also include
  - Comparison of copy numbers
  - Cost analysis
  - Labour and time requirement
- Each assay will also be assessed for its suitability for the detection of *N. perurans* in water
  - 1.2µM filters using pump system designed by CSIRO
- Standardise the methodology for field sampling
  - fully utilise molecular methods to help inform management decisions
  - finely measure potential gains in AGD resistance and altered management practices

Acknowledgements

- Marine Institute
  - Neil Ruane
  - Eve Collins
- GMIT
  - Ian O'Connor
  - Eugene McCarthy
- Vet Aqua International
  - Mar Marcos
  - Hamish Rodger
- CSIRO Staff
  - Mat Cook
  - Richard Taylor
  - Megan Rigby
  - Ben Maynard

Marine Institute
Foras na Mara

NETWORKING INITIATIVE

Support provided by the Marine Institute and funded under the Marine Research Sub-programme by the Irish Government, co-financed by the European Union via the European Regional Development Fund.

EUROPEAN REGIONAL DEVELOPMENT FUND