Investigations on *Paramoeba perurans* and amoebic gill disease at Marine Scotland Science

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Gill Health Initiative 2014 Oslo

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In vitro amoebae cultures

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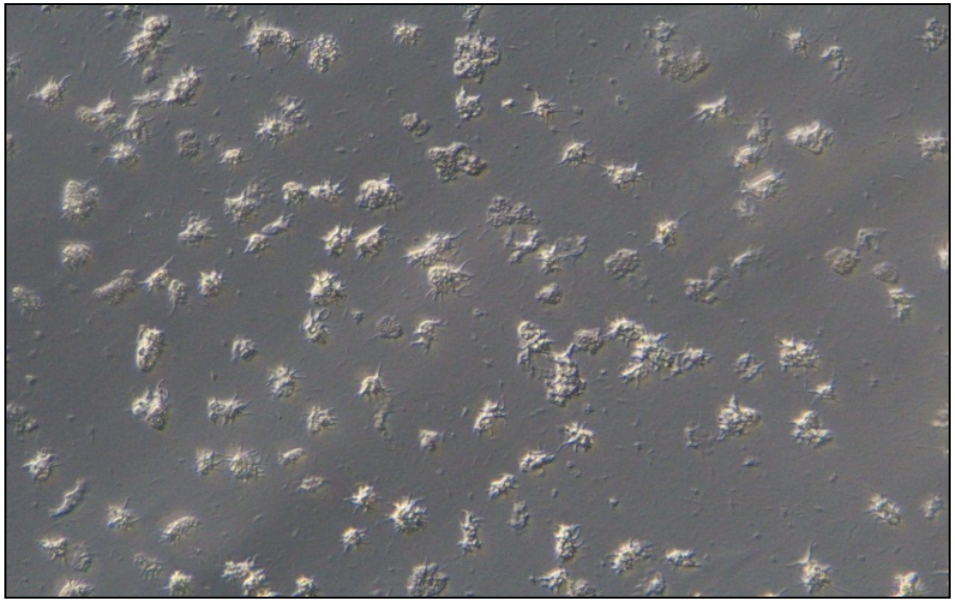
Development of *in vitro* culture for *P. perurans*

- independent supply of parasites for *in vitro* and in vivo experiments
- better standardisation of experimental variables

<u>Generic culture</u>

- cultures established on Nov. 2012 from gill wash from infected farmed salmon
- amoebae grown on malt yeast agar with sea water overlay
- confirmation of species by PCR and QPCR analyses



Attached amoebae on agar surface

In vitro amoebae cultures - clonal

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Clonal cultures of *P. perurans*

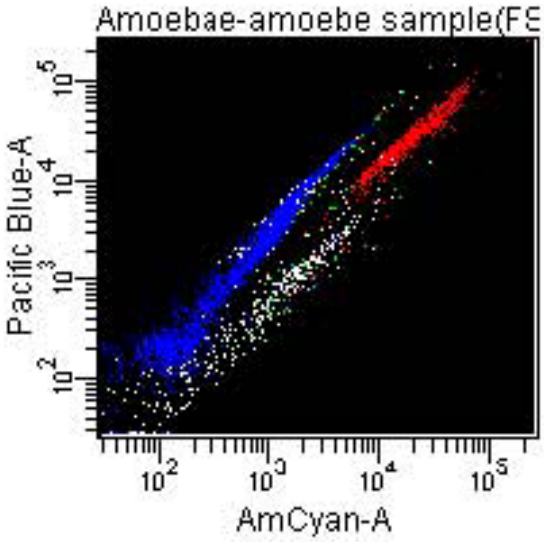
- pure cultures of single species
- less “noise” and greater standardisation for certain experimental approaches
- facilitate identification of, and basis for, intra-specific differences in biological and disease traits

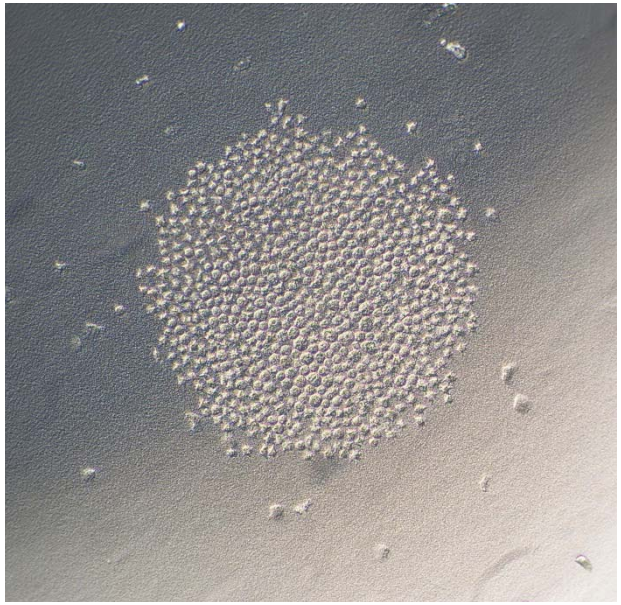
- less representative of field populations and genetic potential within field population

Isolation of clonal cultures using flow cytometry

- isolate single cells into 96 well MYA plates

>50% of single cells generated clones





Clonal population from single cell

Development of challenge model

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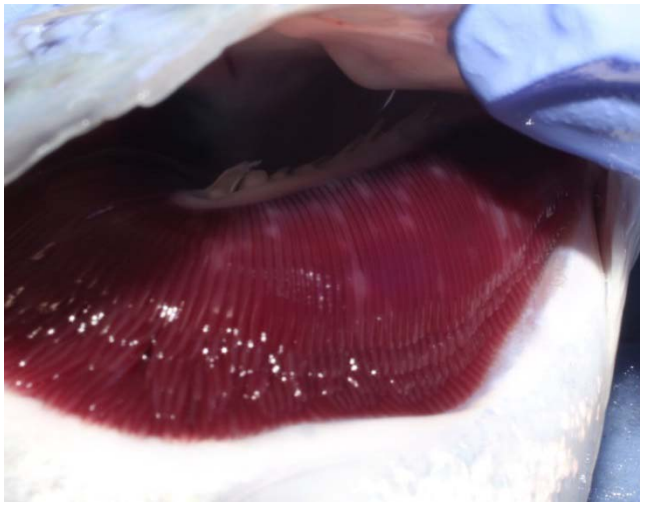
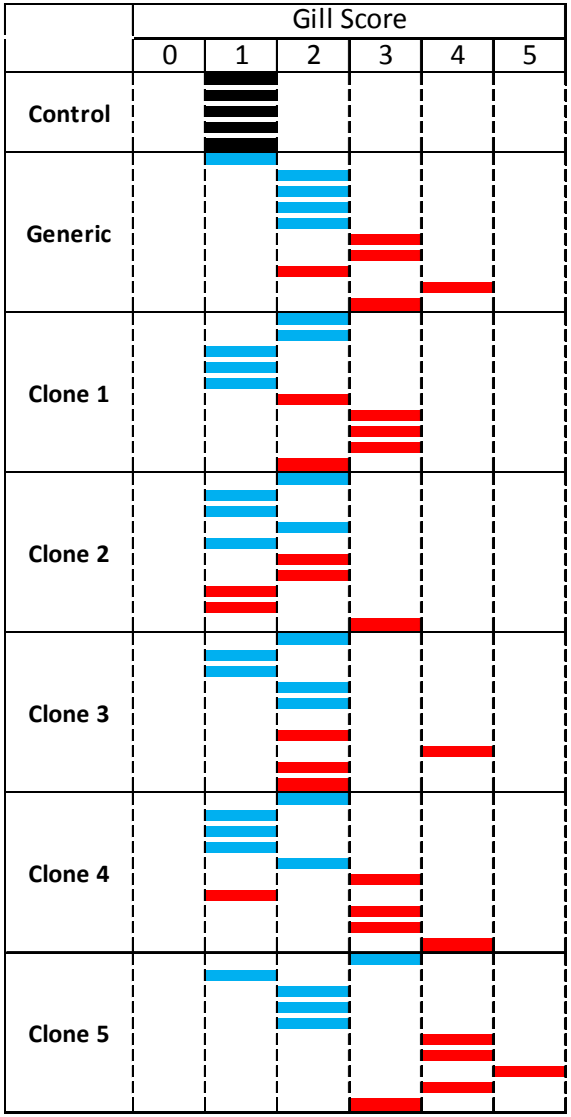
- generic and clonal amoebae cultures
- 5000 & 500/L exposure for 4 hours
- 350-400g fish, 12°C
- bacterial filtrate as negative control
- fish anaesthesised 2 weeks p.i. to observe disease progress
- terminated 3 weeks p.i., all exposed fish developed AGD

- amoebae exposed *in vitro* to MSS222 at 100mg/L for 30 mins
- amoebae stained with vital stain, 1hr , 24hrs post exposure
- no effect on viability observed
- effect on growth rate not evaluated



Challenge model – gill scores



Gill scores based on pathology from H&E stained sections.

- Higher dose resulted in higher gill scores overall
  - 500/L: GS of 1-3 (single incidence of 3)
  - 5000/L: GS of 1-5 (single incidence of 1/5)

- Differences in clone virulence
  - CE6: 2.5 times less likely to induce higher gill damage (SE=0.97, z=-2.41, p=0.02)
  - B8: more likely to induce higher gill damage not statistically significant (z=1.70, p=0.097).

500 amoebae/L

5000 amoebae/L

Challenge Model – data generated

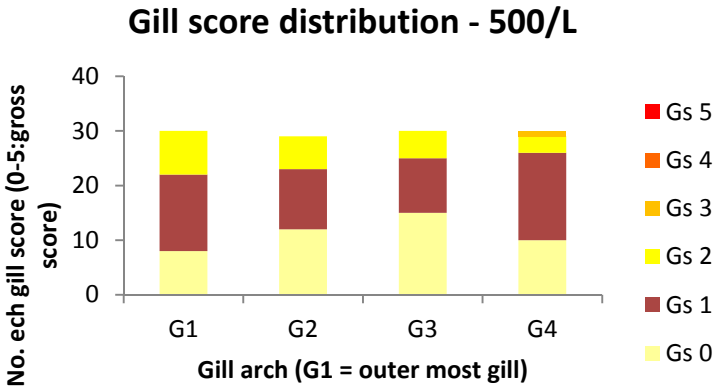
- Gill score based on histopathology analyses of single gill agreed with the average gill score from fresh gills in second gill chamber

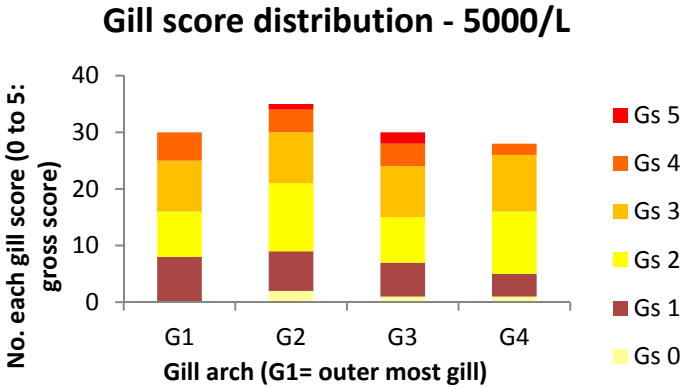
<u>Gross examination of gills</u>

- variability between fish
- variability between gills in individual fish
- variability in location of lesions
- areas with highest occurrence of lesions

- late stage sampling
- aquarium experiment
- salmon species

Gill score distribution among gill arches

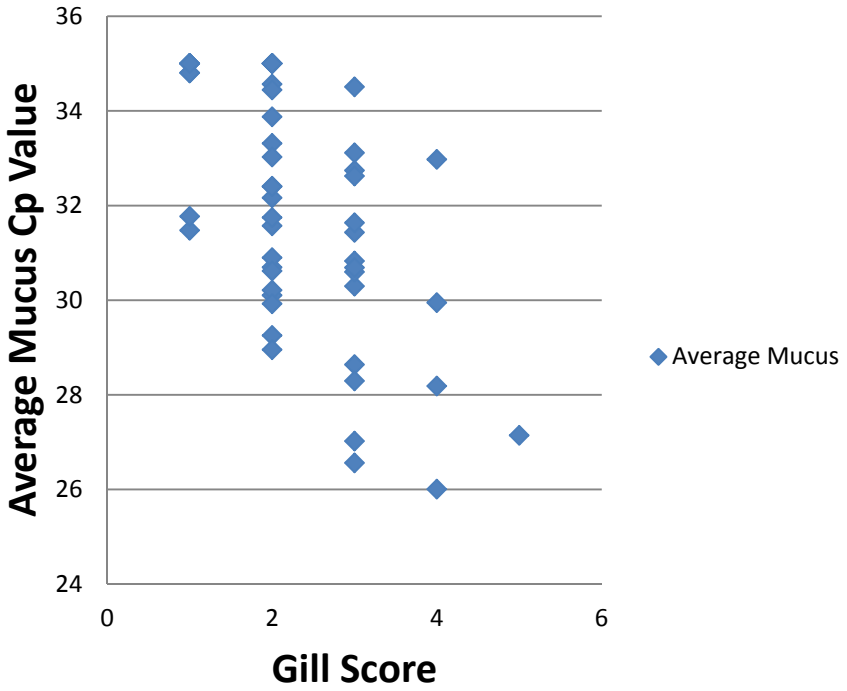




Taylor et al. (2009)

QPCR agreement with Gill score – mucous swabs

- mucous swabs taken from entire gill
- QPCR Ct values compared with gill score
- general trend observed but large overlap in Ct values
- results based on single controlled experiment (field sampling may show even greater variation)



Biophysical properties: salinity and temperature effect on replication

Approach

Temperatures: 2, 4, 8, 11, 15, 18, °C

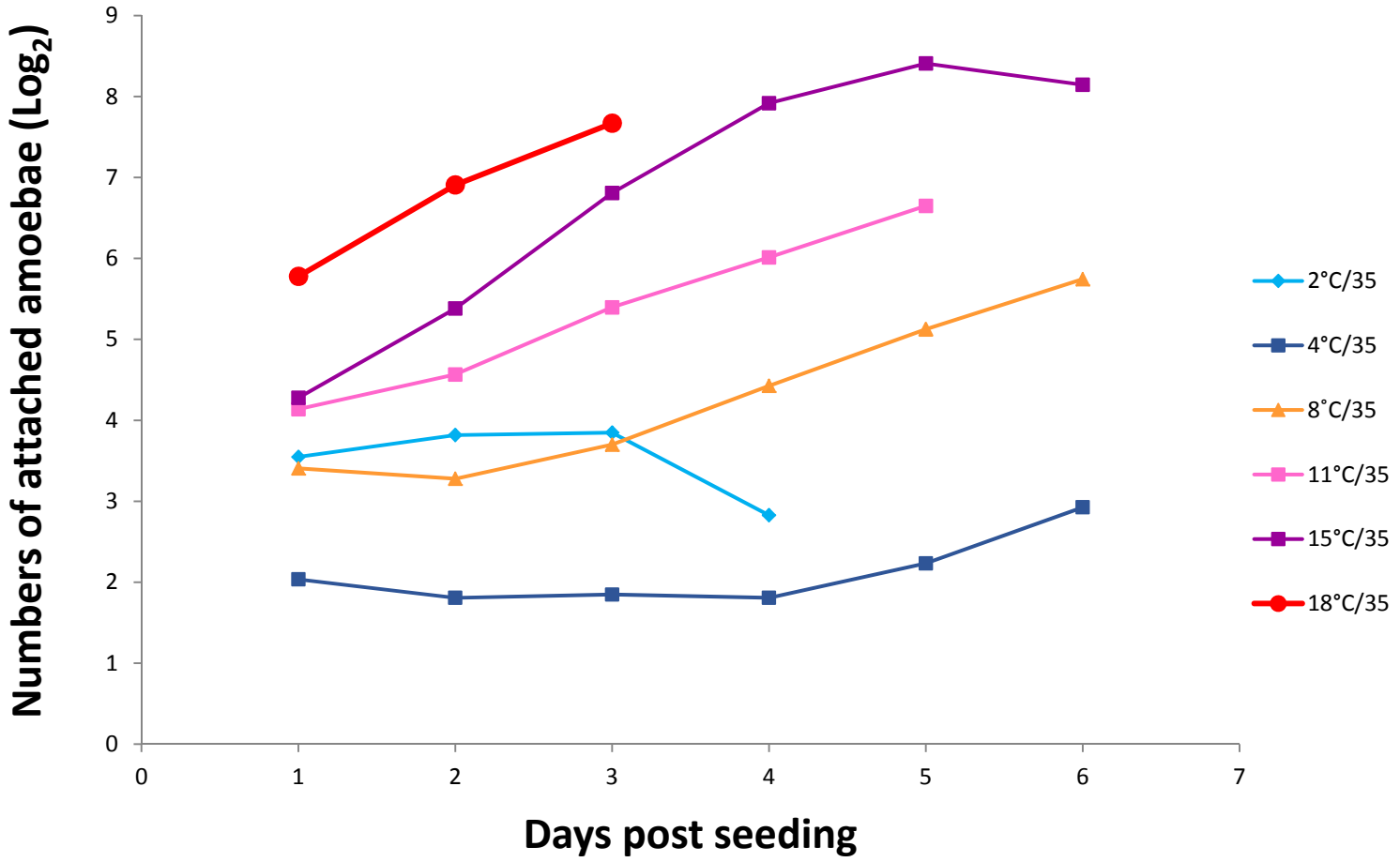
Salinities: 0, 3, 5, 15, 20, 25, 30, 35, 40, 45, 50

In vitro "generic" culture

- temperatures randomised as blocks (n= 24)
- salinities fully randomised (n=3 for each salinity)
- amoebae acclimatised for 48 hours at temperature
- amoebae seeded into T25 culture flasks with MYA
- inactivated bacteria added as food source every two days
- counts taken daily for 9 days and day 12, 15 post seeding
    - 100ul culture suspension, neutral red, counted
    - 10 photos across transect of flask for counting attached amoebae

Biophysical properties: salinity and temperature effect on replication

Population growth curves: variable temperature/fixed salinity



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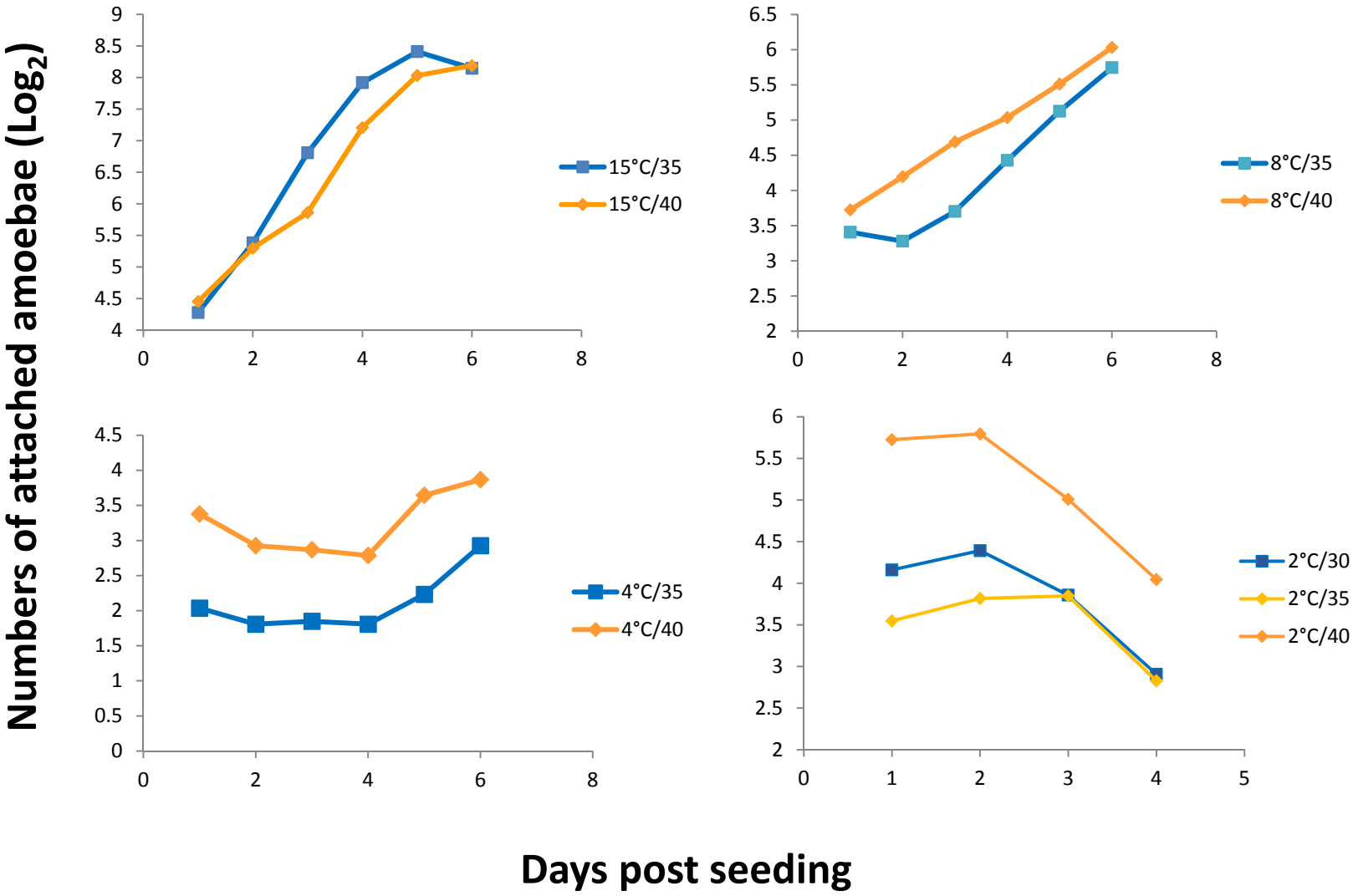

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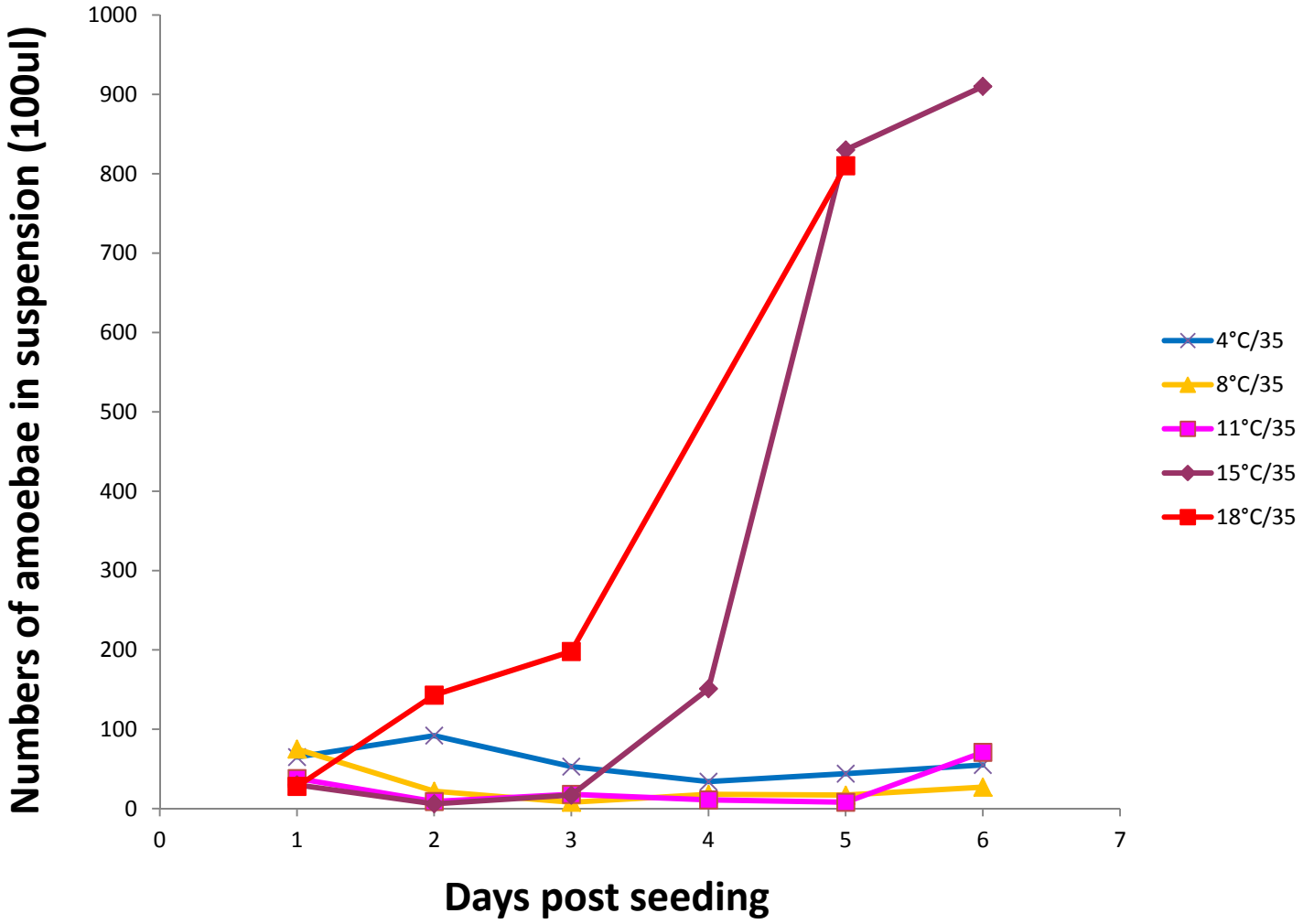

Screening wild reservoirs and environmental samples

Presence of *P. perurans*/AGD in wild fish populations

- Background prevalence against which to compare more specific groups
  - wild fish associated with farms
  - migrating species
  - wild salmonids

MSS cruises

International Bottom Trawl Surveys (north east and west coasts)

- West coast bottom trawl, Mar. 2013, 1150 fish, 23 sites.
- North Sea and Shetland bottom trawl, Feb. 2013, 1200 fish, 24 sites

Sampling

- 50 fish per trawl site, numbers of each species sampled based on proportional representation
- section of outer gill sampled into 100% ethanol
- QPCR (+ BOXTO) analyses for *P. perurans,* and for host 18S rDNA (quality control check)
- a low number of amplification products obtained with slightly different melting temperatures (BOXTO) to *P. perurans* control– awaiting sequencing for identification

Molecular epidemiology: samples and markers

Sampling plan agreed with Scottish industry.
7 different regions representing all of Scotland

Genetic Markers

- data mining of CSIRO transcriptome information has provided candidate sequence data for Cytochrome Oxidase 1 (CO1) mitochondrial gene(s)

- two sequences provided: approximately 70% similarity at the nucleotide level.

- PCR assays designed and successful amplification of PCR products from Scottish clonal *P. perurans* cultures for one of the assays

- initial partial sequence data indicate similarity to CSIRO transcriptome sequence data

In vivo Hydrogen Peroxide experiment

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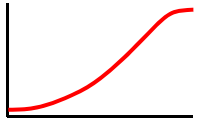
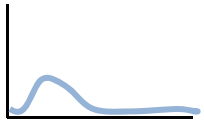
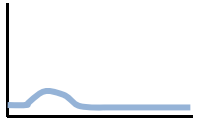
Aquarium challenge – when is the best time to treat AGD?
Gill pathology
Pathogen load

AGD and HP	AMOEBA	Hydrogen peroxide	Treat D3	Treat D9	Treat D12	Treat D15	Treat D18	Sample each at fixed time after treatment	
AGD and FW		Freshwater	Treat D3	Treat D9	Treat D12	Treat D15	Treat D18	Sample each at fixed time after treatment	
AGD alone		No treatment	Sample D3	Sample D9	Sample D12	Sample D15	Sample D18		
Treatments alone	No AMOEBA	Hydrogen peroxide	Treat D3					Sample at fixed time after treatment	
		Freshwater	Treat D3					Sample at fixed time after treatment	
Baseline gill damage		No treatment	Sample D3	Sample D9	Sample D12	Sample D15	Sample D18	Sample at D30	

Acknowledgements

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&

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