Development and adaptation of an *in vitro* rainbow trout gill model for use as an alternative to live fish studies

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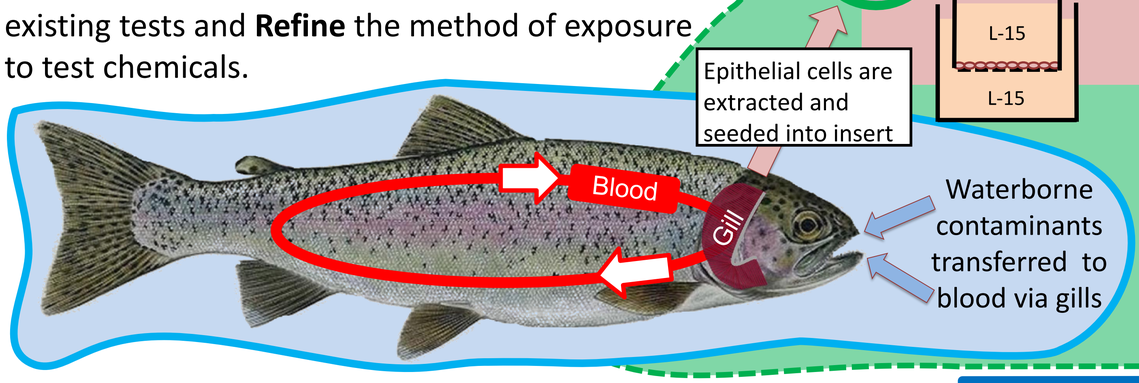
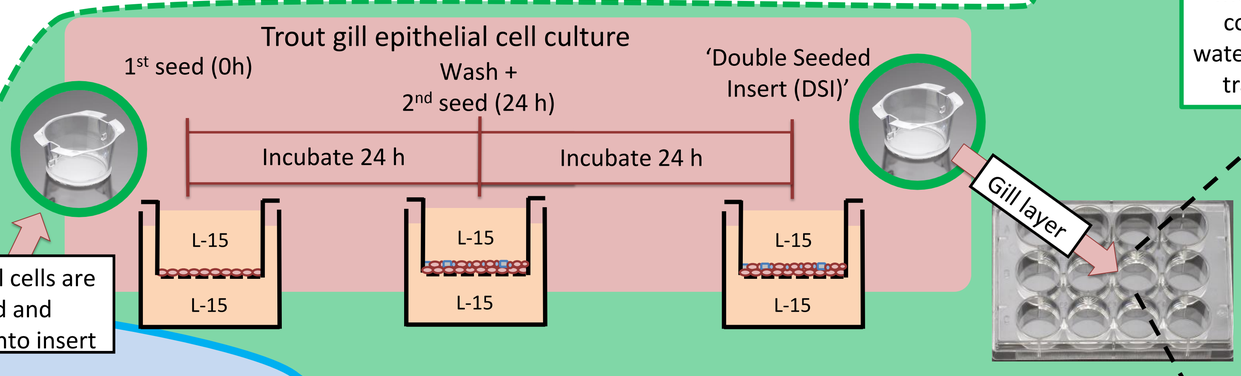
Introduction

In line the 3Rs, our work involves the development and validation of novel, reliable *in vitro* fish methods as an alternative to *in vivo* studies. The rainbow trout (*Oncorhynchus mykiss*) gill has a long history of cell and tissue models, which have developed over time from a single to a double seeding of cells in flasks and then microplate inserts[2-5]. One issue with the existing gill model for toxicological testing is the relatively short (~2 days) duration of viability after apical exposure to water. If this time could be extended, potential applications for the model would be increased e.g. to include longer term chronic testing to better represent current *in vivo* ecotoxicological tests. Such a model would **Reduce** the number of fish used in existing tests and **Refine** the method of exposure to test chemicals.



The aim of the current study was therefore to investigate the effect of different culture methods on the gill model viability and to maximise its lifespan and functionality after apical water addition.

O. mykiss in vivo /in vitro comparison

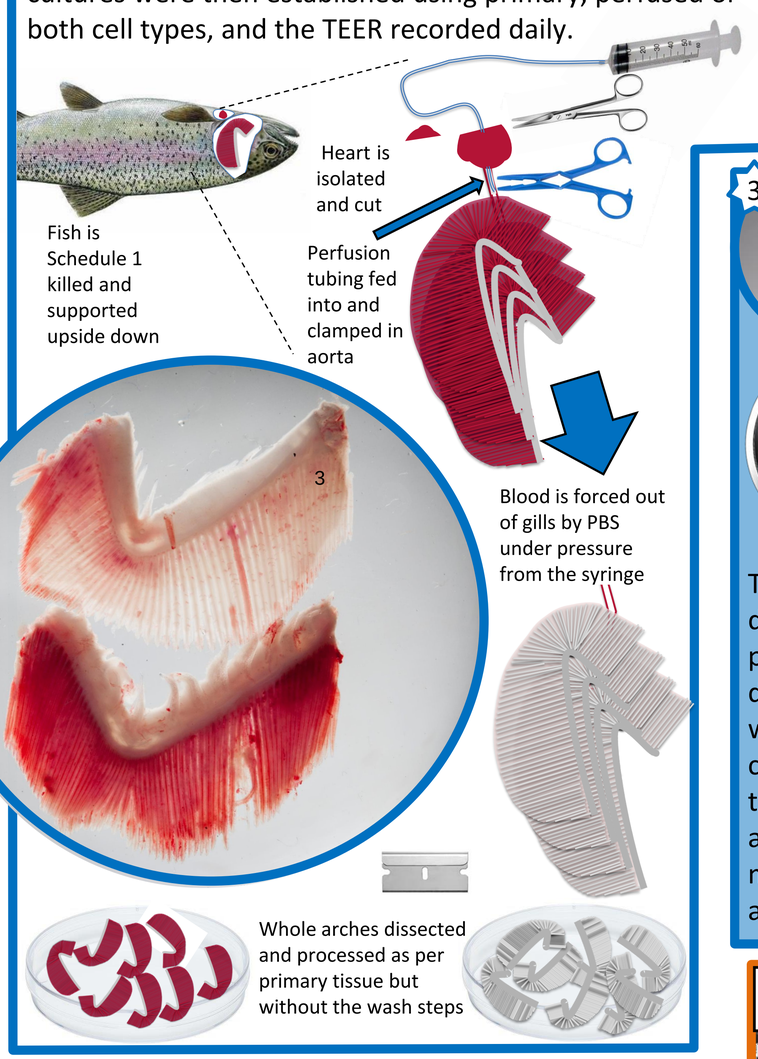


Materials & Methods

We have investigated various ideas to improve the time the model remains viable after addition of apical water. The methods reported here include: 1 Culture serum type, 2 Pre-perfusion of gills, and 3 Use of different membrane materials.

1 Cells were grown in symmetrical culture (L-15 media on both sides) with either 5% Fetal Bovine Serum (FBS) or Trout Serum (TS) before replacing the media on the apical side with artificial freshwater (USEPA moderately hard water).

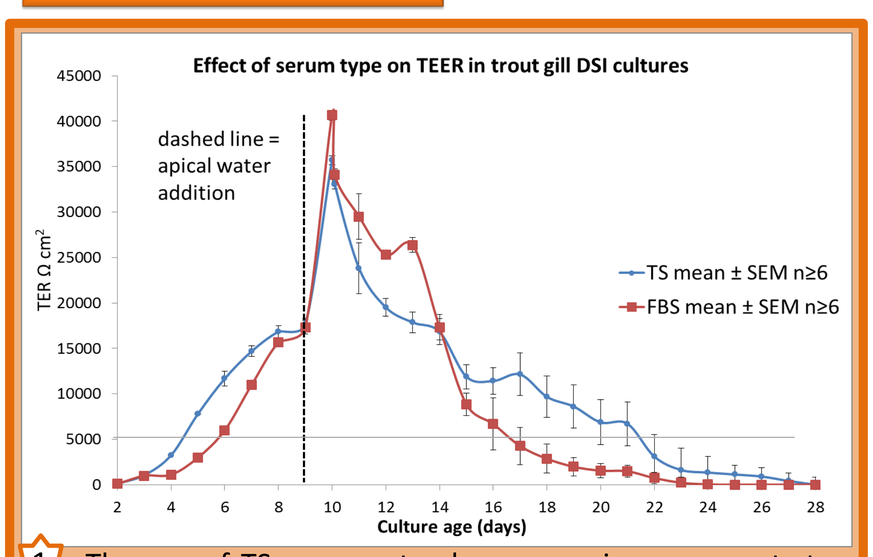
2 The gills were perfused *in situ* through the aorta with PBS via syringe to remove blood cells from filaments. The perfused cells then went straight into the trypsin stage. DSI cultures were then established using primary, perfused or both cell types, and the TEER recorded daily.



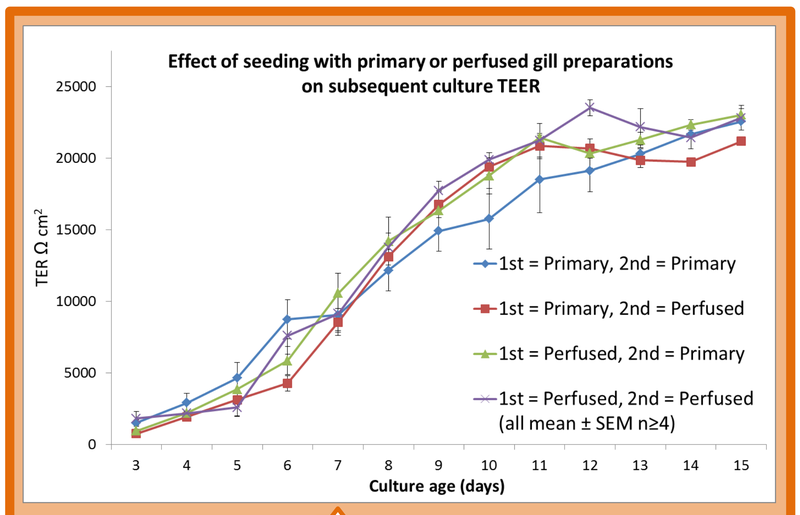
The **Trans-Epithelial Electrical Resistance (TEER)** across a cell culture can be measured by a WPI EVOM2 'chopstick' electrode. It is a standard measure of the cell membrane 'tightness' and can be interpreted as a measure of culture quality[6]

3 Three different plastic polymer or pore density membranes were tested against the control for TEER through time. SEM imaging was also used to assess membrane appearance and cell attachment.

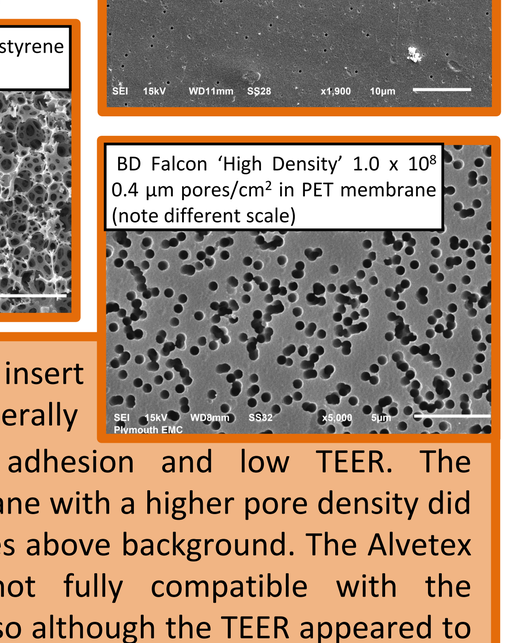
Results & Discussion



1 The use of TS appears to show some improvements to TEER compared to FBS cultures. TS cultures reach 5KΩ cm^2 (a standard threshold) a day sooner than FBS under symmetrical conditions and remain above 5KΩ cm^2 for several days more after water addition. FBS cultures appear to reach a higher maximal TER immediately after water addition. Both of the culture types are extending far beyond the ~48 hours previously reported.[7]

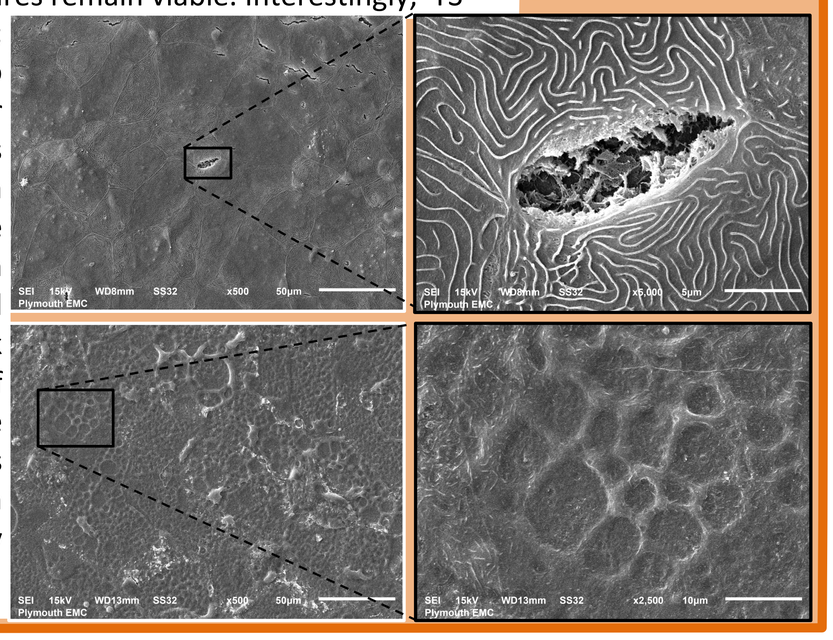


2 We tested primary and/or perfused culture preparations for the two cell seeds. NSD was observed between any of the 4 combinations of cell seeding were found. Although no apparent benefit for TEER, the perfusion method does reduce the protocol time for culture preparation by limiting the wash stages and allows earlier seeding of cells.



3 The alternative insert membrane types generally displayed low cell adhesion and low TEER. The identical PET membrane with a higher pore density did not attain TEER values above background. The Alvetex membrane was not fully compatible with the chopstick electrode, so although the TEER appeared to be low, it could not be accurately measured. Only the Transwell (polycarbonate) membrane produced similar growth to the control (data not shown). It is unclear why the cells did not attach, but the seeding densities might require optimisation for specific insert types, particularly the 3D scaffold-like Alvetex. We are also yet to try collagen-coated inserts from the major suppliers which are successfully used in mammalian in vitro cell culture.[8]

SEM analysis of surface ultrastructure of a typical culture for FBS or TS at Day 5 post water addition (PWA; top) reveals microridges, tight junctions and varying cell types. After ten days PWA only TS cultures remain viable. Interestingly, TS cultures (e.g. bottom; Day 18) appear to exhibit depressed, or coated microridges with development of a larger scale ridge structure, similar in scale to *in vivo* apical crypts. Further work on characterisation of this version of the model and explore its use in longer term exposures is currently being explored.



References

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