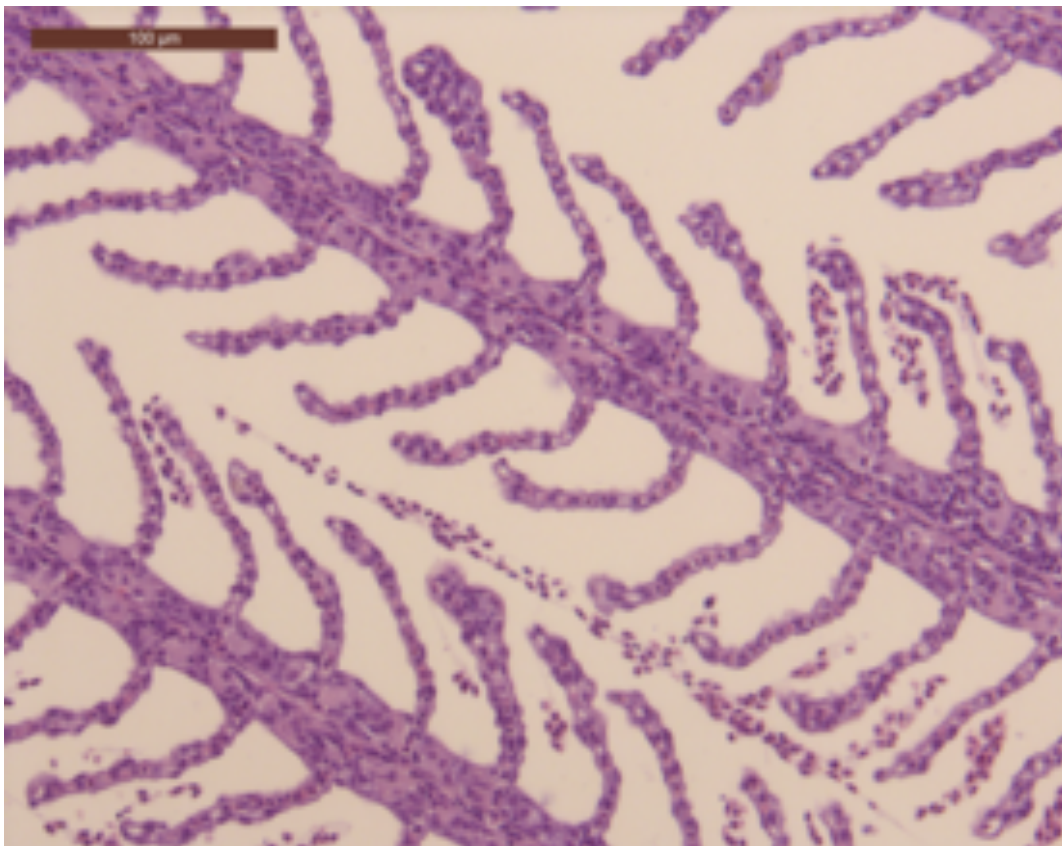


AGD control and disinfection in cleanerfish

Part 2: Challenge trial



Norwegian Institute for Water Research

REPORT

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<p>Summary</p> <p>Juvenile ballan wrasse, <i>Labrus bergylta</i> were exposed to a <i>Neoparamoeba perurans</i> polyculture either UV irradiated at a low (2mJ cm⁻²) or high (20mJ cm⁻²) dose of UV radiation from a medium pressure UV lamp in a beam collimeter. Control fish consisted of un-exposed fish and fish exposed to non-irradiated <i>Neoparamoeba perurans</i>. Over the subsequent 6 weeks of maintenance, amoebic gill disease (AGD) only developed in the non-irradiated amoeba challenged group with a gross gill score peaking at 3 with 100% prevalence. Similarly, only the non-irradiated amoeba group showed characteristic AGD pathology and was the only group in which <i>Neoparamoeba perurans</i> were detected by PCR.</p> <p>This study indicated that UV irradiation of <i>Neoparamoeba perurans</i> is sufficient to prevent the onset of infection if irradiation exceeds 2 mJ cm⁻²</p>

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AGD control and disinfection in cleanerfish
Part 2: Challenge trial

Preface

The experiments described in this report were conducted at NIVAs Marine Research Station at Solbergstrand and Oslo and Bergen as part of the AGD Control-Disinfection of Cleanerfish (ACDC) project financed through the RFF Vest and Marine Harvest ASA.

Oslo, 16th September 2016



Mark D. Powell

Summary

Juvenile ballan wrasse, *Labrus bergylta* were exposed to a *Neoparamoeba perurans* polyculture either UV irradiated at a low (2mJ cm^{-2}) or high (20mJ cm^{-2}) dose of UV radiation from a medium pressure UV lamp in a beam collimeter. Control fish consisted of un-exposed fish and fish exposed to non-irradiated *Neoparamoeba perurans*. Over the subsequent 6 weeks of maintenance, amoebic gill disease (AGD) only developed in the non-irradiated amoeba challenged group with a gross gill score peaking at 3 with 100% prevalence. Similarly, only the non-irradiated amoeba group showed characteristic AGD pathology and was the only group in which *Neoparamoeba perurans* were detected by PCR.

This study indicated that UV irradiation of *Neoparamoeba perurans* is sufficient to prevent the onset of infection if irradiation exceeds 2 mJ cm^{-2} .

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1 Introduction

Amoebic gill disease is well recognized as a major disease of Atlantic salmon caused by the amphizoic parasitic amoeba *Neoparamoeba perurans* with a high cost for control (Powell et al. 2008). However, salmonids are not the only susceptible fish species. Recently, disease outbreaks in ballan wrasse (*Labrus bergyllia*) (Karlsbakk et al. 2013), cultured as a cleanerfish species for the biological control of sealice in Atlantic salmon culture, have occurred. This raises two important issues. Firstly that AGD has the potential for significant impact in wrasse culture facilities, and secondly that affected cleanerfish may transmit amoebae to Atlantic salmon if covertly infected fish are stocked into sea cages for biological sealice control.

Following a previous in vitro study into the effects of UV and ozone disinfection on *Neoparamoeba perurans*, it was concluded that although amoebae were responding to UV irradiation by exhibiting stressed morphologies (rounding up), they recovered and motile polymorphic trophozoites were seen, although cell division was inhibited and cultures did not grow (Wennberg and Powell 2015). Eventhough the amoeba cultures failed to grow, in vitro assays do not test whether cultures are capable of causing disease and attaching to the gills of fish. Therefore an in vivo challenge experiment was designed to test the hypothesis that UV irradiation at a low dose or high dose (2 or 20 mJ cm⁻² respectively) could inhibit gill colonization and the subsequent development of AGD in ballan wrasse, *Labrus bergyllia*.

2 Materials and methods

Fish and challenge facilities

Juvenile female ballan wrasse, *Labrus bergylta* were transferred to Solbergstrand Marine Research Station where they were housed in duplicate 400L tanks (50 fish per tank) supplied with flowing seawater at 12°C with minimum oxygen limits of 80% saturation. Fish were fed a commercial pelleted diet (Ottaheime) consistent with that provided at the hatchery of origin as well as supplemented with cooked prawns. Prior to exposure to amoebae, 2 fish from each of the 8 tanks were removed, killed with an overdose of MS222 (Metacain 100 mg L⁻¹) and sampled as described below.

UV exposure of amoebae cultures

Polyculture of amoebae including *Neoparamoeba perurans* isolated from an active amoebic gill disease infection of Atlantic salmon at Solbergstrand Marine Research. The resulting culture was maintained in a flat bottom culture flask with malt-yeast broth (MY-broth; 0.01% malt extract, 0.01% yeast extract, filtered seawater) by changing 90-100% of the medium once a week. The water phase was used to spike new culture bottles (Nunc EasYFlask 175 cm², Nunclon Delta Surface). All cultures were incubated at 16°C ($\pm 1^\circ\text{C}$). Subcultures were harvested 7-14 days after inoculation, when most of the cells had left the bottle surface and formed a star-shaped floating stage (Wennberg and Powell 2015).

The cultures were poured into 50ml centrifuge tubes (VWR) and centrifuged at 3 000xg for 10 min. The supernatants were discarded and the pellets from 3-5 tubes were collected in one tube and re-suspended in sterile seawater. The centrifugation was repeated, the supernatant discarded and the pellet re-suspended in sterile seawater. This procedure reduced the concentration of dissolved organic carbon and increased the UV-transmittance. The washed cultures were kept on ice until used within 4 hours.

The UV-transmission (UV-t) of the sample was measured at 254 nm before the experiment using UVT15 PV photometer (HF Scientific inc), and the UV intensity of the UV lamps at 254 nm were measured at 5 points on the exposure area to calculate the average UV intensity of each experiment using a UVX radiometer (UVP inc). Exposures were carried out using a medium pressure UV lamp collimated beam set-up using 10 seconds exposure for low UV dose and 90 sec exposure for high UV dose. A petri dish with a magnetic rod was added 50 ml of culture just before UV exposure. The suspension was stirred gently by the magnetic bar during exposure. UV doses was calculated according to Bolton and Linden (2003). A 10-fold dilution series was made in a 96 well plate with culture medium and monitored for growth of amoebae for 5 days using an inverted microscope Olympus IX71, 20x10 magnifications. After UV exposure, the cultures were stored at 4°C overnight before used in the challenge trial.

Challenge protocol and sampling

Prior to exposure to amoebae, 2 fish from each of the 8 tanks were removed, killed with an overdose of MS222 (Metacain 100 mg L⁻¹) and sampled as described below.

The water supply to each tank was stopped and additional oxygen added to maintain O₂ levels above the minimum. Amoeba culture was then added to introduce an equivalent of 1000 suspended amoeba cells per litre of water. Fish were maintained for 1 hour before the water flow was reinstated. Negative controls consisted of tanks to which no amoebae were added.

Weekly for 6 weeks, 5 fish from each tank (10 per amoeba challenge) were removed and killed by an overdose of MS222. Fish were weighed and measured and a caudal blood sample taken and analysed by ISTAT. Gills were scored for gross pathology (white patches) using the same scheme as that for Atlantic salmon (from Taylor et al 2009) and one arch placed in RNALater for PCR analysis and the remaining arches into neutral buffered formalin for routine histological examination. PCR analysis was undertaken directly from the samples using a DNA-based assay specific for *Neoparamoeba perurans*.

3 Results

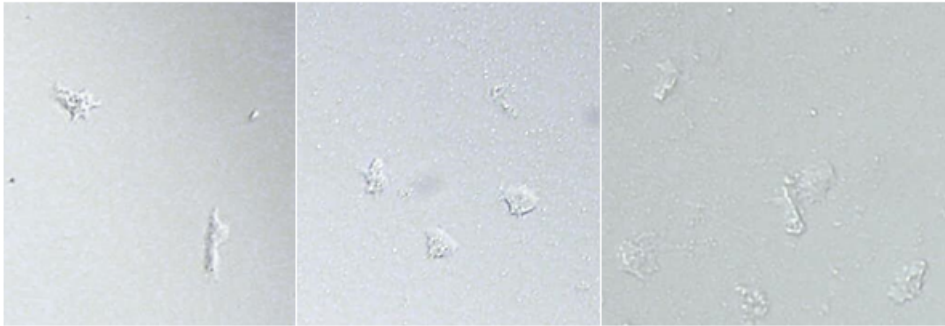
The UV-t of the cultures was 50.5%, resulting in UV-doses of 2 mJ cm⁻² for the 10second exposure and 20 mJ cm⁻² for the 90 seconds exposure. The amoebae culture responded to the UV treatment in the same manner as in the previous experiment described in (Wennberg and Powell 2015) (Fig 1). The amoebae receiving high UV dose looked deformed without pseudopods the same day of exposure, and had no growth the following 5 days. The amoebae exposed to low UV dose looked unaffected immediately after exposure and the next 5 days following exposure, but with no growth. The non-exposed control culture had good growth with more than a doubling of numbers on day 1 after exposure.

There was a progressive increase in gill score in only one of the groups challenged with amoebae, the group challenged with non-irradiated amoebae (ANOVA $F_{6,69}$ 16.94 P value <0.001)(Fig 2). Neither the amoebae irradiated at the low or high doses induced any significant gill score. Similarly, 3 weeks post-challenge, the non-irradiated amoeba group had 100 prevalence of gill scores (Fig 3). Histologically there was minor background pathology in most groups (Fig 4). However, only non-irradiated amoeba group had pathology consistent with amoebic gill disease (Fig. 5). Using real time PCR, it appeared that only the group challenged with non-irradiated amoebae were positive for *Neoparamoeba perurans* DNA which reached a prevalence of 50% 6 weeks post challenge. None of the other groups (negative control, low UV dose or high UV dose) showed any positive signal for *Neoparamoeba perurans* DNA (Fig 6).

Control non-irradiated
D0

d1

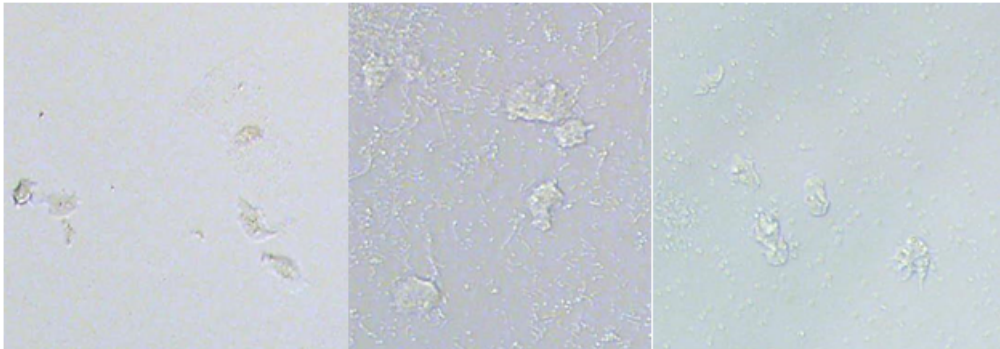
d5



UV exposed low dose (2mJ/cm²)
D0

d1

d5



UV exposure high dose (20mJ/cm²)
D0

d1

d5

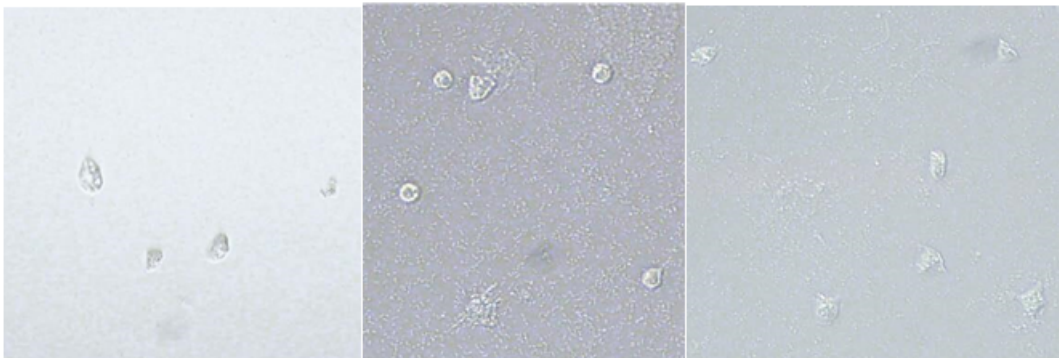


Figure 1. Morphology of amoebae 1-2 h (D0), and 1 and 5 days post-exposure to UV irradiation at a low dose (2 mJ cm⁻²) or high dose (20 mJ cm⁻²).

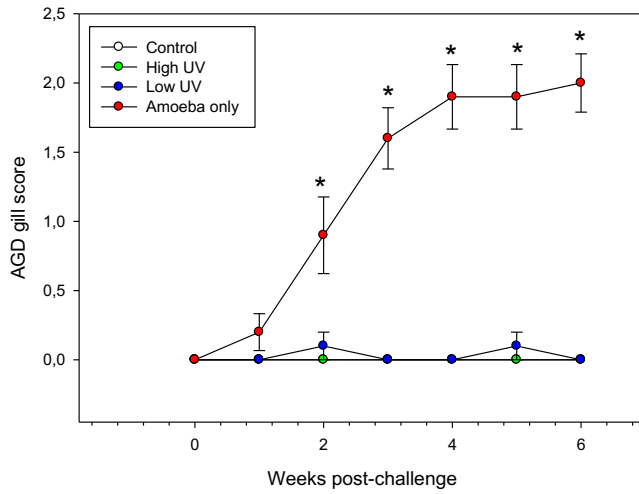


Figure 2 Mean AGD gross gill score (\pm SEM) of ballan wrasse challenged with amoebae irradiated with either a nigh UV dose, low UV dose or non-irradiated amoebae (amoeba only). Controls represent fish not challenged with any amoebae.

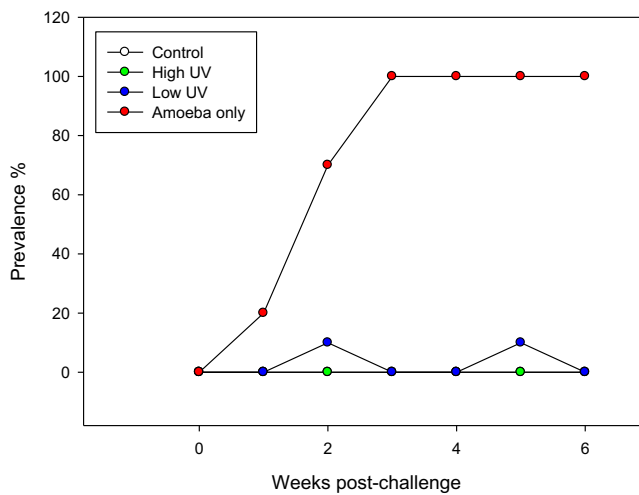


Figure 3 Prevalence of positive gross gill scores in ballan wrasse challenged with amoebae irradiated with either a nigh UV dose, low UV dose or non-irradiated amoebae (amoeba only) . Controls represent fish not challenged with any amoebae.

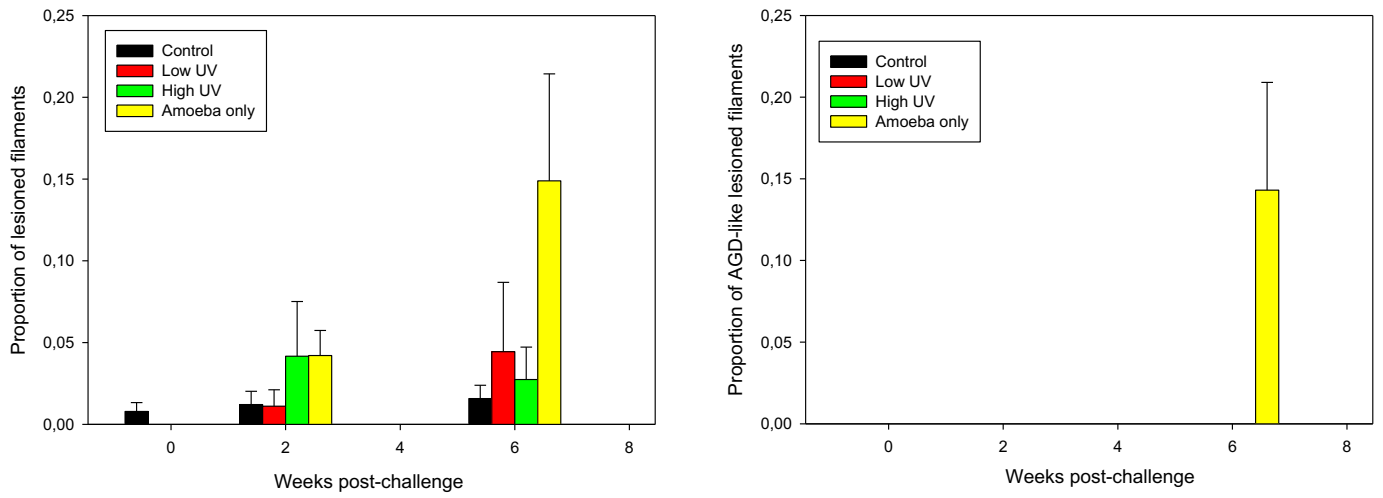


Figure 4. Mean proportions (+ SEM) of lesioned gill filaments and AGD-like lesions (Fig 4) in ballan wrasse challenged with amoebae irradiated with either a high UV dose, low UV dose or non-irradiated amoebae (amoeba only). Controls represent fish not challenged with any amoebae.

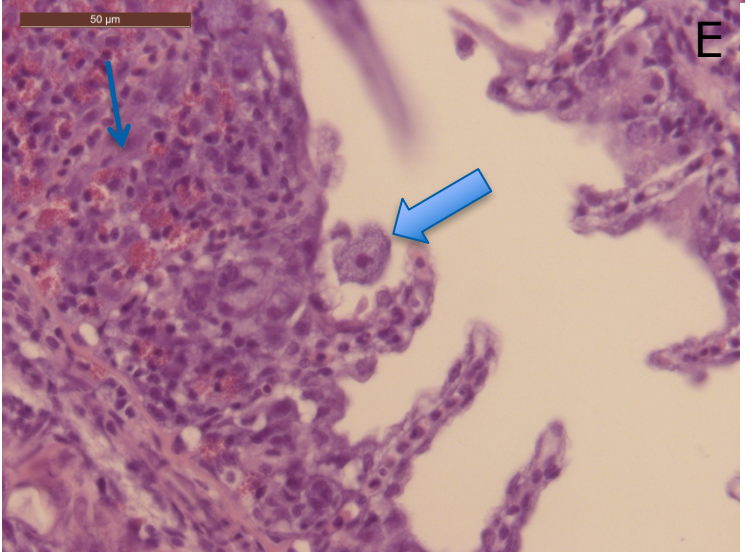
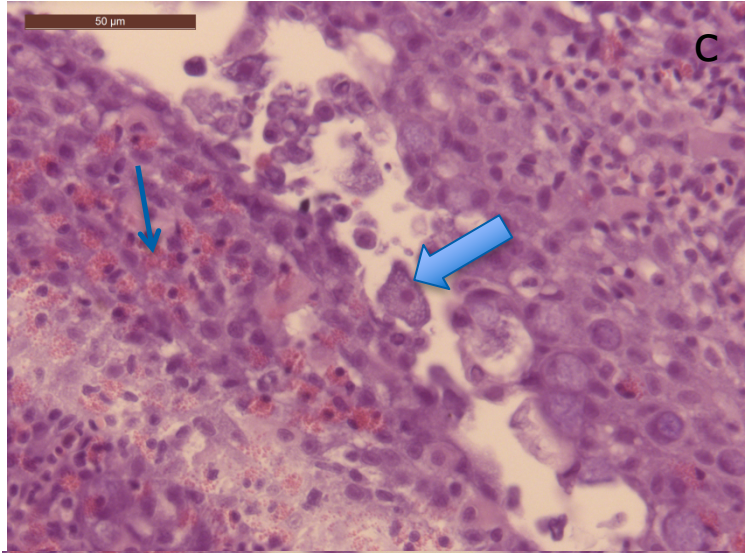
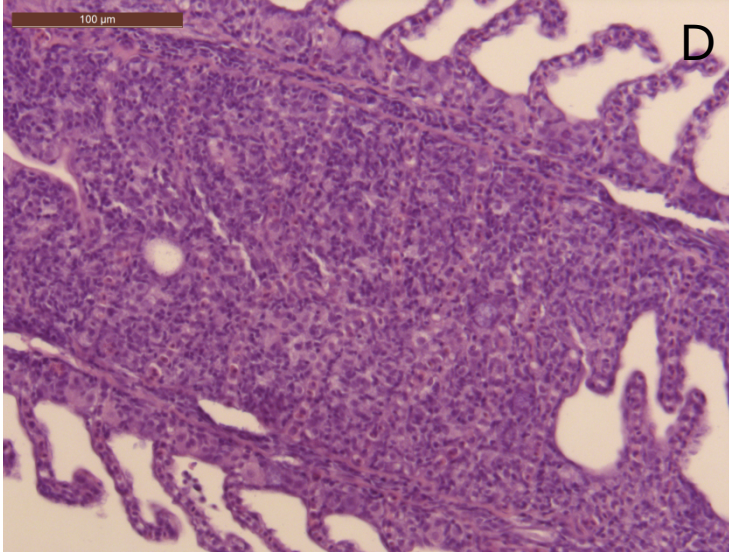
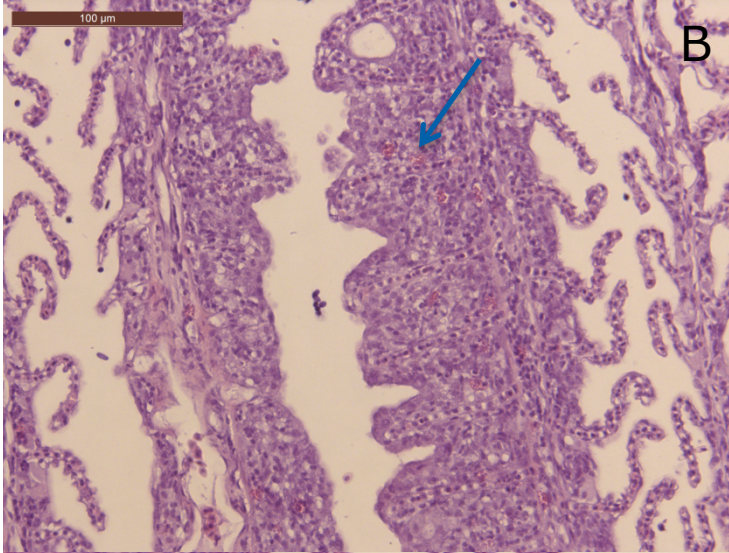
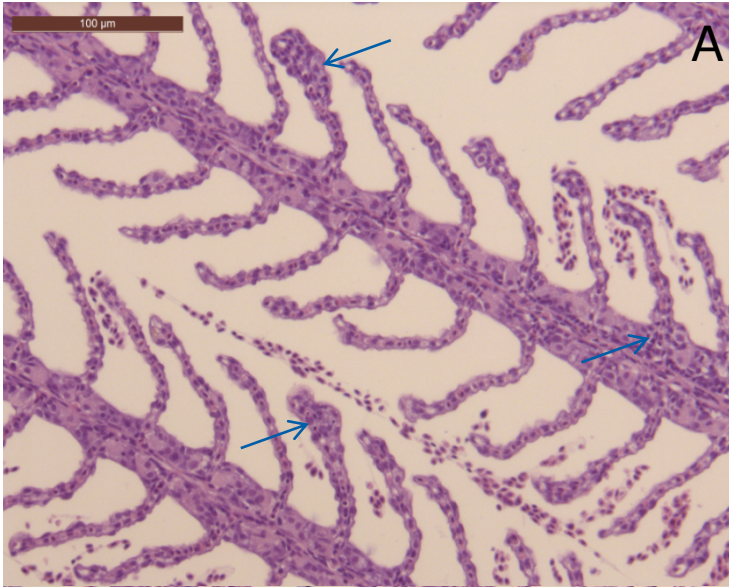


Figure 5. A Background pathology in non-AGD affected ballan wrasse showing hyperplastic and inflamed individual lamellae and filamental inflammation (fine arrows). B-E Hyperplastic filamental epithelium associated with AGD in ballan wrasse. Eosinophils in the hyperplastic plaque associated with AGD (thin arrow) and the presence of a *Neoparamoeba*-associated with the epithelial surface of the gill (thick arrow). Stain H&E.

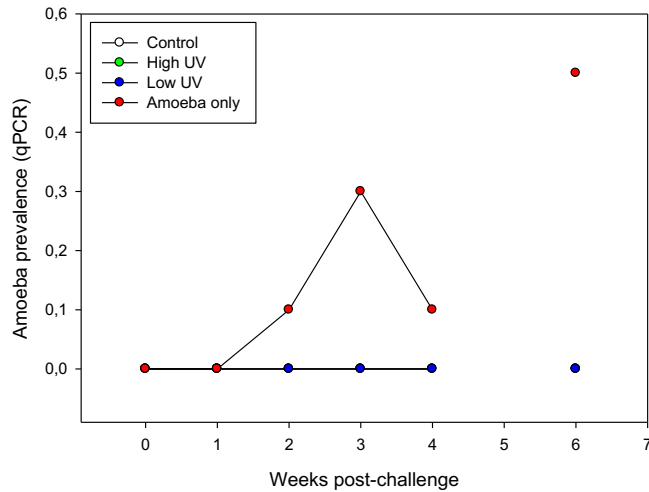


Figure 6. Prevalence of *Neoparamoeba perurans* –positive ballan wrasse using a DNA based qPCR challenged with amoebae irradiated with either a high UV dose, low UV dose or non-irradiated amoebae (amoeba only) . Controls represent fish not challenged with any amoebae

4 Discussion

There was clearly no infection in the control fish that were not challenged with *Neoparamoeba perurans*. Similarly, prior irradiation with either a low UV dose or high UV dose was unable to induce disease in ballan wrasse. On the other hand, when fish were challenged with the same amoebae but not irradiated with UV, AGD developed in all challenged fish. The best indicator of pathology in this study was the gross gill score, followed by histology. Although there was some background pathology as described above, the majority of fish showing AHD-like gill pathology were exclusively from the non-irradiated amoeba only group. Similarly this same group was the only group to show positive results using qPCR. However, not all AGD-positive (by gill score or histology) appeared positive by PCR and this is most likely to a limited sensitivity of the PCR assay used in this study.

AGD presentation, particularly at low gill score can vary in its presentation quite extensively, especially in non-salmonid species. When this is coupled with a technique such as histology where determination and quantitation of disease is made using a 3-5 μm tissue section, the presence of amoebae or lesions can be missed giving the appearance of false negative results. Similarly, a low sensitivity of the DNA-based PCR test used in this study also is likely to have yielded a number of false negative results. Further on-going investigation will be undertaken to increase the qPCR assay sensitivity and further characterize the pathology and disease progression in ballan wrasse.

In conclusion, based upon the evidence provided using gross gill pathology, histology and *Neoparamoeba perurans*-specific qPCR in this pilot project, it is clear that irradiation of *Neoparamoeba perurans* at a low UV dose (2 mJ cm⁻²) was sufficient to prevent the development of AGD in ballan wrasse over a 6 week challenge period. However, it is recommended that UV doses exceeding this value be used for the effective control of AGD in ballan wrasse culture facilities.

5 Acknowledgement

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