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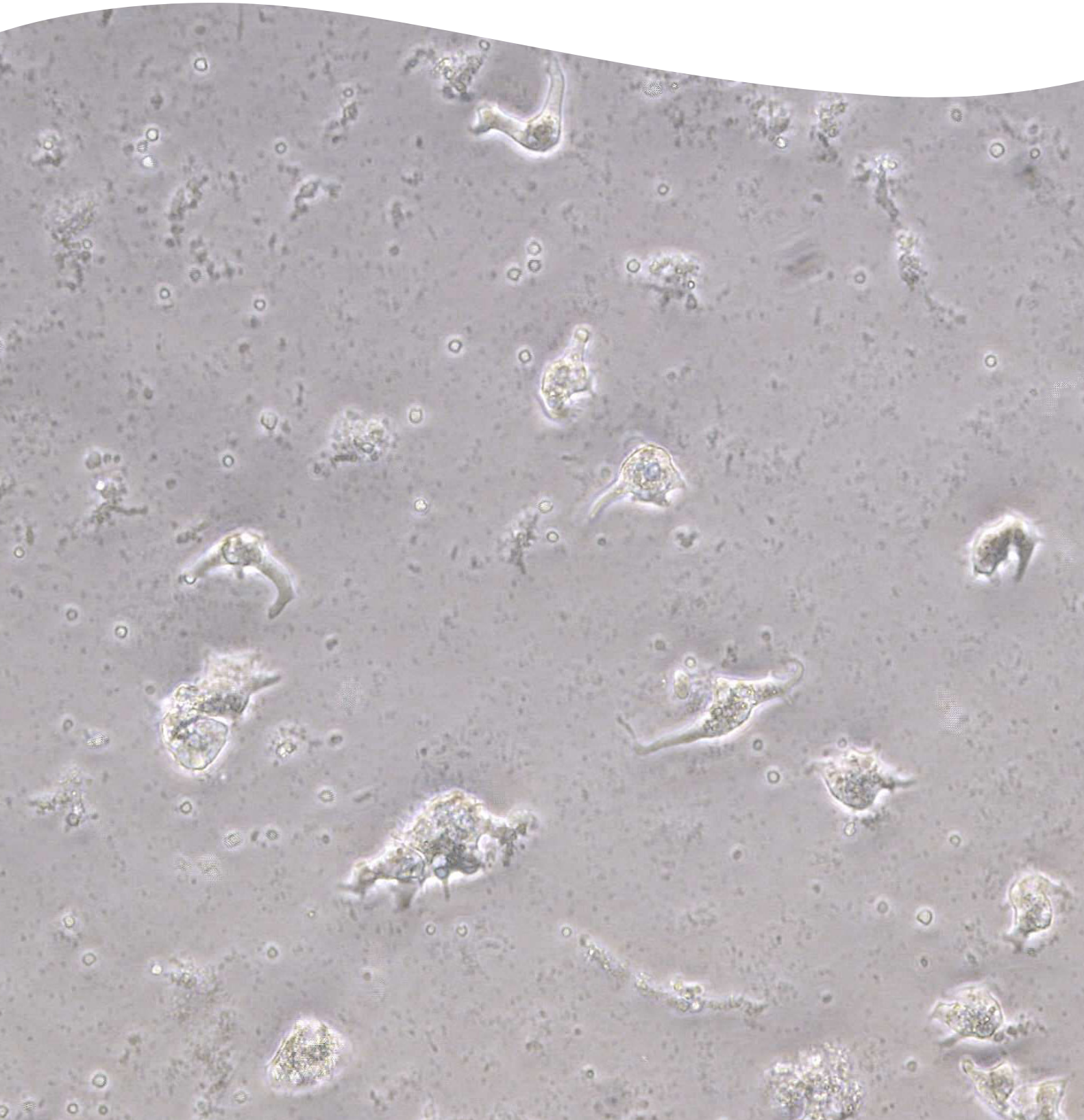
Disinfection of Paramoebae perurans with UV and ozone
In situ dose-response testing



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REPORT

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Abstract

Lab cultures of the amoebae *Paramoebae perurans* was exposed to UV irradiance at varying durations or ozonated seawater with varying residual oxidant (TRO) concentrations. The response to the treatment was observed as both changes in morphology and the ability to reproduce. UV exposures were performed with both a low and a medium pressure UV lamp in a collimated beam set-up. The amoebae lost their ability to reproduce even at the shortest UV exposure time (5 sec), both with low and medium pressure UV lamps (UV_{254}-doses of 4 and 2 mJ/cm^2, respectively). The amoebae had much higher resistance towards ozonated seawater than expected. They retained their ability to reproduce and seemed unaffected by the treatment even at the highest dose tested (25 mgCl_2*min/L).

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Preface

The experiments described in this report were conducted at NIVAs lab in Oslo as part of the project AGD Control-Disinfection of Cleanerfish (AC-DC) financed by RFF Vest and Marine Harvest.

Oslo, 15.09.2015

Aina Charlotte Wennberg

Contents

Summary

Lab cultures of the amoebae *Paramoebae perurans* was exposed to UV irradiance at varying durations or ozonated seawater with varying residual oxidant (TRO) concentrations. The response to the treatment was observed as both changes in morphology and the ability to reproduce.

UV exposures were performed with both a low and a medium pressure UV lamp in a collimated beam set-up. The amoebae lost their ability to reproduce even at the shortest UV exposure time (5 sec), both with low and medium pressure UV lamps (UV_{254}-doses of 4 and 2 mJ/cm^2, respectively). However, the amoebae looked unaffected by the low UV doses and had active pseudopods and vesicles for several days after exposure. At UV-doses of 4 to 64 mJ/cm^2 and 5 to 10 mJ/cm^2 with low and medium pressure lamps respectively, the amoebae looked unaffected the first day, but rolled up into an inactive form after a few days. At doses higher than 66 mJ/cm^2 and 11 mJ/cm^2 with low and medium pressure lamps respectively, the amoebae were visibly affected without pseudopods and vesicles at the same day as the exposure. The cells were destroyed at doses higher than 48 mJ/cm^2 with medium pressure lamps.

The amoebae had much higher resistance towards ozonated seawater than expected. They retained their ability to reproduce and seemed unaffected by the treatment even at the highest dose tested (25 $mgCl_2*min/L$).

Sammendrag

Labkultur av amøben *Paramoebae perurans* ble eksponert for UV-lys med varierende eksponeringstid eller ozonert sjøvann med varierende restoksidantkonsentrasjon (TRO). Responsen på behandlingen ble observert som både forandring i morfologi, evne til reproduksjon og ødeleggelse av cellene.

UV eksponering ble utført med både en lavtrykks lampe og en mellomtrykks lampe i et «collimated beam» oppsett. Ved UV-eksponering mistet amøbene evnen til reproduksjon allerede ved korteste eksponeringstid (5 sekunder) med både lavtrykk og mellomtrykkslampen (UV_{254}-doser på hhv 4 og 2 mJ/cm^2). Amøbene så derimot upåvirket ut og hadde aktive pseudopodier og vesikkeltransport i flere dager etter eksponering. Med doser på 4 til 64 mJ/cm^2 for lavtrykk og 5 til 10 mJ/cm^2 for mellomtrykk så amøbene først upåvirket ut, for deretter å trekke seg sammen og bli inaktive etter noen dager. Ved doser over 66 mJ/cm^2 for lavtrykk og 11 mJ/cm^2 for mellomtrykk var amøbene tydelig skadet uten pseudopodier og vesikler, og ved doser over 48 mJ/cm^2 for mellomtrykk ble cellene ødelagt.

Amøbene viste mye større resistens mot ozonert sjøvann enn forventet. Selv ved høyeste dose benyttet (25 $mgCl_2*min/L$) beholdt de fortsatt evnen til reproduksjon og virket upåvirket av behandlingen.

1. Introduction

Amoebic gill disease (AGD) poses a significant threat to western Norwegian aquaculture. The causative agent (*Paramoeba perurans*) has been identified as the cosmopolitan cause of AGD (Young et al., 2007) and was first identified in fish from the western part of Norway in 2006 (Steinum et al., 2008). In Ireland and Scotland, AGD has had a severe impact to the salmon aquaculture industry. Since 2009 the number and distribution of European AGD outbreaks indicate a northward spread and in 2014 69 outbreaks of AGD were recorded extending along the Norwegian coast as far north as Nord-Trønderlag (http://www.kyst.no/Havbruk/?article_id=111131).

The current methods for control of AGD are frequent freshwater or hydrogen peroxide baths. The use of hydrogen peroxide to treat amoebic gill disease and lice infestations in farmed salmon has tripled in Norway during the last year, from 2538 tons in 2012 to 8262 tons in 2013 (www.fhi.no, Norwegian Public Health Institute). These treatments have proven logistically challenging and costly, with significant impact on fish appetite, growth and survival. Additional tools for AGD control are urgently required to prevent the re-occurrence of disease outbreaks in cleanerfish hatcheries as well as other facilities at risk such as post-smolt production where salmon smolt are on-grown in closed or semi-closed facilities using seawater to produce a larger fish for stocking into open net-pens.

The best way to prevent loss of fish during treatment is to prevent the fish from being infected. In closed systems, the water can be disinfected and the pathogens removed before entering the fish tanks. It is however a challenge to scale the disinfection to the water flows needed. UV and ozone separate or in combination are the most common water disinfections used in Norwegian aquaculture today, but their effect on *P. perurans* is not known.

This study was designed to find the doses necessary for inactivating the amoebae using UV or ozone treatments.

2. Materials and methods

2.1 Culture conditions and harvesting/preparations

The amoebae culture was kindly provided from ILAB in Bergen, where the amoebae were isolated after an exposure experiment. The 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 50-95% of the medium once or twice a week. The water phase was used to spike new culture bottles (Nunc EasYFlask 75cm^2 or 175 cm^2, Nunclon Delta Surface). All cultures were incubated at 16°C (±1°C). Subcultures were harvested 4-14 days after inoculation, when most of the cells had left the bottle surface and formed a star-shaped floating stage.

The cultures were poured into 50ml centrifuge tubes (VWR) and centrifuged at 3 000xg for 10 minutes. The supernatants were discarded and the pellets from 3-5 tubes where 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 was done to reduce the concentration of dissolved organic carbon and increase the UV-t. The washed cultures were kept on ice until used within 4 hours.

2.2 Experimental set-up UV

Four experiments were done with both medium and low pressure UV lamps in a collimated beam set-up, using 4 to 6 different exposure times and one non-exposure control for each experiment; see **Table 1** for exposure times. The UV-transmission (UV-t) of the sample was measured at 254nm before the experiment using UVT15 PV photometer (HF Scientific inc), and the UV intensity of the UV lamps at 254nm were measured at 5 points on the exposure area to calculate the average UV intensity of each experiment using a UVX radiometer (UVP inc).

Table 1. Exposure times [sec] to UV irradiance of *P. perurans* in four separate experiments each using a low and a medium pressure UV lamp in a collimated beam set-up.

Sample no	Exposure time low pressure [sec]				Exposure time medium pressure [sec]			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4
1	60	10	5	5	60	15	5	5
2	120	20	15	10	240	30	15	15
3	210	40	20	20	480	60	30	30
4	300	80	80	80	720	90	90	90
5		150	150	150			150	150
6			210	210			240	240

A petri dish with a magnetic rod was added 25 ml of culture just before UV exposure. The suspension was stirred gently by the magnetic bar during exposure. Immediately after exposure 1 ml culture was withdrawn and the exposure continued for the next dose. The water depth of the petri dish was less than 1 cm to reduce the effect of the low UV-t of the samples. The petri dish was placed in a larger petri dish filled with iced water to prevent the culture from warming up too much during UV irradiance. The temperature of the culture was monitored.

2.3 Experimental set-up ozone

Four to five different ozone concentrations were tested in addition to a control with no added ozone in three experiments with a contact time of 3 minutes per concentration tested. The ozone concentration was measured as total residual oxidants (TRO) as mg Cl_2/L using the total chlorine DPD method (HACH). The intended start concentration of ozone for each experiment is shown in Table 2.

Table 2. Intended start concentration of ozone measured as mg/L TRO in three experiments.

Sample no	Intended start concentration (mg/L TRO)		
	Exp. 1	Exp. 2	Exp. 3
1	**0.4**	**0.5**	**2**
2	0.8	1	3
3	1.2	2	4
4	1.8	3	5
5			10

Ozonated seawater was prepared fresh within 30 min before use by bubbling ozone gas from a Wedeco ozone generator through filtrated seawater from 60m depth to give an absorbed ozone concentration measured as 15-42 mg/L TRO. The ozonated seawater was diluted with seawater that would result in the intended start concentrations given in Table 2. The ozonated seawater (45mL) was added 5ml of amoebae culture. The concentration of TRO was measured in the culture at 30 sec after addition of culture and after 3 min. A solution of sodium thiosulfate (10g/L) was added to neutralize the ozone after 3 min exposure time. The cultures were kept in a water bath and the temperature during each exposure was monitored.

2.4 Quantifying amoebae

A 10-fold dilution series was made in 2ml tubes: 1ml undiluted, 0.1mL + 0.9mL MY broth, etc. from 10^{-1} to 10^{-5} diluted. 100µL of each dilution was added to 5 wells each on a 96 well plate already filled with 100µL MY broth. Three wells were added 20µL of undiluted sample to be counted immediately. The dilution plate was monitored for amoebae growth for up to 7 days using an inverted microscope Olympus IX71, 20x10 magnifications. Concentration was calculated based on MPN table B.6 in NS-EN ISO 8199:2007.

2.5 Calculation of exposure doses

The exposure dose for each experiment was calculated as: concentration X time = dose

For UV irradiance, the average UV exposure of the sample must be calculated as the UV intensity (I) will not be the same at the surface of the water film as at the bottom. A magnetic rod was used to mix the water during irradiation to reduce the variance of exposure and justify the use of an average UV dose based on the depth of the water film and the UV-t of the sample.

Mean UV intensity: $I = \frac{I_0}{L} \int_{L \to 0}^{L=0.573} \frac{(1-e^{-AL})}{AL}\, dL$
I=intensity
A= absorbance 254nm, $A = -\log(\frac{UV-t\%}{100})$
L= light path/water depth

The UVdose [mJ/cm²] was then calculated as $UV_{dose} = I \times t$,
Were t is the exposure time [sec].

The organic content of the culture consumes oxidants so that the TRO concentration during the ozone exposure experiments would decrease over time. Thus the ozone dose was calculated based on the tree

TRO measurements done for each sample: prior to addition of culture (adjusted for dilution), 30 sec after addition of culture and after 3 minutes.

Ozone dose[mg Cl_2*min/L] $= \left(\frac{TRO_{start} \times 0.9 + TRO_{30s}}{2}\right) \times 0.5min + \left(\frac{TRO_{30s} + TRO_{3min}}{2}\right) \times 2.5min$

2.6 Control of culture identity by PCR

A PCR was run on the culture from the control in each experiment to confirm that *P. perurans* was present in all experiments. From each well plate with the control culture 600µl was transferred to a 1.5ml centrifuge tube and centrifuged at 20 000g for 1 min and the pellet re-suspended in 100µl deionized milliQ water and stored at -20°C for two weeks. The tubes were heated 10 min at 95°C to disrupt the cells before used as template in the PCR set up.

A PCR was run using PCR primers and a modified protocol from Bridle et al (2010). PCR reaction mix for total volume of 20µl SooFast Evagreen super mix 10x conc, 0.5µM primers, 2µL template.

3. Results

3.1 Amoebae morphology observations

The aim of this study was to establish dose response curves of the effect of UV and ozone towards *P. perurans*. However, as the result of the experiments showed either full inactivation or no effect to growth, the morphological responses to the treatments were described instead of the reduction of viable cells. The use of vital or exclusions stains as trypan blue and neutral red was also tested without success. Thus, the responses to treatments are described as capability to reproduce and as change in morphology non-quantitatively. **Figure 1** and **Figure 2** show examples of amoebae from non-exposed cultures that would multiply with a doubling time of approximately 20 hours at the given culturing conditions. **Figure 1** shows the floating stages of the cultured amoebae with extended pseudopods, while **Figure 2** shows amoebae attached to the bottom of the culture flasks with various forms of extended pseudopods and various numbers and sizes of vesicles inside the amoebae. **Figure 3** shows typical amoebae shapes a few days after UV irradiance. The cells have balled up and there are few or no extended pseudopods or vesicles.

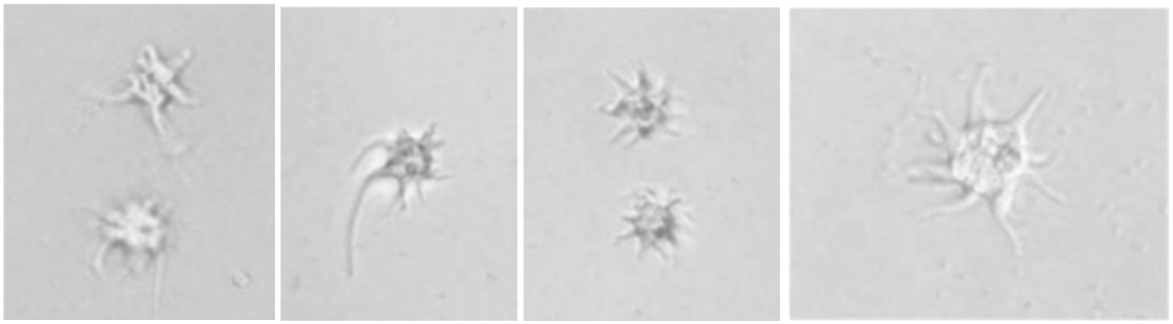

Figure 1. Floating stage of non-exposed amoebae (Photo: A. C. Wennberg)

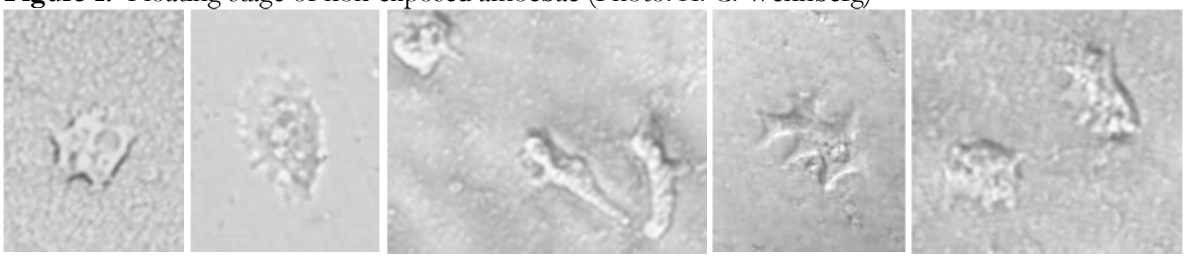

Figure 2. Attached form of non-exposed amoebae with active pseudopods and multiple vesicles. (Photo: A. C. Wennberg)

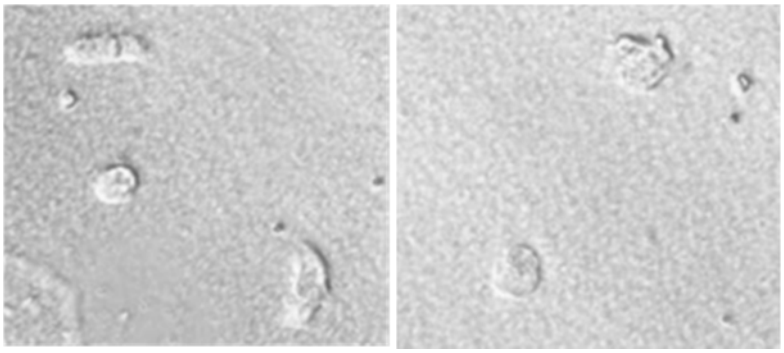

Figure 3. Stressed or damaged amoebae that has balled up several days after UV exposure (Photo: A. C. Wennberg)

3.2 UV low pressure exposures

The experiments were done with amoebae cultures with concentrations of 1.6 to 8.4 x10^3 cells/mL and with a UV-t and temperature as shown in **Table 3**. The UV doses were calculated based on the formula in chapter 2.5 and the exposure times in **Table 1**.

Table 3. UV doses and experimental conditions for low pressure UV experiments

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Mean UV intensity lamp [µW/cm²]	743	832	814	840
UV-t sample	85 %	81 %	85 %	87 %
Temperature [°C]	7.5-15.0	4.9-9.6	5.6-16.0	14.1-19.6
Sample no	**UV_{254} dose [mJ/cm²]**			
1	44	8	4	4
2	88	16	12	8
3	154	33	16	17
4	220	66	64	67
5		123	121	125
6			169	175

None of the amoebae exposed to UV irradiance would multiply, meaning they were inactivated at even the lowest UV dose tested. However, the control sample treated in exactly the same way as the test samples behaved normal with a doubling time of approximately 20 hours, confirming the results. At the lowest tested UV dose (4 mJ/cm²), the amoebae seemed morphologically unaffected and had still active pseudopods and vesicles four to seven days after exposure. However, at higher doses, the amoebae balled up and seemed to be inactive a few days after exposure. At doses around 66 mJ/cm², most of the amoebae looked stressed and balled up just a few hours after exposure, then they would be active for the first day or two, attaching to the bottom of the well, before they would round up again. However, this was not the case each time, so the doses around 64 to 66 mJ/cm² seemed to be a tipping point to where acute affects occurred. See **Table 4** for a summary of the doses and effects.

Table 4. Acute and long term effects on *P. perurans* of exposure to low pressure UV doses.

UV-doses [mJ/cm²]	Acute effects (same day as exposure)	Long term effects (4-7 days after exposure)
0 (control)	None. Cells are polymorphic with active pseudopods and vesicle transport.	Exponential growth with approx. 20 hours doubling time.
4	None. No difference from control	No growth, or maximum one doubling. Morphology same as day 0.
4-64	None. No difference from control	Small and round or partly rounded up without active pseudopods or vesicles.
66-220	Round cells with short or no pseudopods	Small and round or partly rounded up without active pseudopods or vesicles.

3.3 UV medium pressure exposures

The experiments were done with amoebae concentrations of 1.4 to 8.4 x10^3 cells/mL and with a UV-t and temperature as shown in **Table 5**. The UV doses were calculated based on the formula in chapter 2.5 and the exposure times in **Table 1**.

Table 5. UV doses and experimental conditions for medium pressure UV experiments

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Mean UV_{254} intensity lamp [µW/cm²]	302	367	339	323
UV-t sample	85 %	82 %	84 %	86 %
Temperature [°C]	8.6-16.6	5.5-7.5	9.6-12.2	6.8-11.0
Sample no		**UV_{254} dose (mJ/cm²)**		
1	18	5	2	2
2	72	11	5	5
3	144	22	10	10
4	215	33	30	29
5			50	48
6			80	77

The same effect as seen with low pressure UV was also seen with medium pressure UV irradiance except for at lower UV_{254}-doses, see **Table 6**. However, the highest doses tested (48-215 mJ/cm²) damaged the cells enough for them to disintegrate within a few days. The tipping point for this effect was around 48-50 mJ/cm².

Table 6. Acute and long term effects on *P. perurans* of exposure to medium pressure UV doses.

UV-doses [mJ/cm²]	Acute effects (same day as exposure)	Long term effects (4-7 days after exposure)
0 (control)	None. Cells are polymorphic with active pseudopods and vesicle transport.	Exponential growth with approx. 20 hours doubling time.
2-5	None. No difference from control	No growth, or maximum one doubling. Morphology same as day 0.
5-10	None. No difference from control	Small and round or partly rounded up without active pseudopods or vesicles.
11-50	Round cells with short or no pseudopods	Small and round or partly rounded up without active pseudopods or vesicles.
48-215	Small round and possible damaged	Most cells are disintegrated

3.4 Ozone exposure

The temperature, TRO consumption and doses used in the ozone exposure experiments are listed in **Table 7**.

None of the ozone doses tested in these experiments had any significant effect on the amoebae cultures; see **Table 8** and **Figure 4.** MPN quantification showed overlapping 95% confidence intervals for all samples, indicating that there was no significant reduction in viable amoebae in these experiments. However, at the highest ozone concentration tested, the amoebae looked rounded up and stressed a few hours after exposure, even if they reproduced the next days.

Table 7. Temperature, TRO consumption and ozone doses calculated based on TRO measurements for three ozone exposure experiments.

		Exp. 1	Exp. 2	Exp. 3
Mean TRO consumption 30s		0.2	0.1	0.4
Mean TRO consumption 30s to 3 min		0.1	0.5	0.4
Temperature [°C]		4.0-12.8	12.2-13.5	9.2-10.0
Dose [mg Cl_2 *min/L]	Sample no 1	**1.3**	**1.5**	**3.1**
	Sample no 2	**1.8**	**3.3**	**7.4**
	Sample no 3	**3.1**	**4.2**	**9.0**
	Sample no 4	**3.3**	**7.8**	**13**
	Sample no 5			**25**

Table 8. Concentration of viable amoebae after ozone experiments quantified by most probable number (MPN) method including 95% confidence intervals.

	Exp. 1			Exp. 2			Exp. 3		
	MPN	min	max	**MPN**	min	max	**MPN**	min	max
Control	**1 300**	350	3 000	**920**	300	3 200	**920**	300	3 200
Sample no 1	**2 800**	900	8 500	**920**	300	3 200	**1 600**	640	5 800
Sample no 2	**1 300**	350	3 000	**920**	300	3 200	**540**	180	1 400
Sample no 3	**490**	170	1 300	**540**	180	1 400	**920**	300	3 200
Sample no 4	**700**	230	1 700	**280**	90	850	**130**	35	300
Sample no 5							**350**	120	1 000

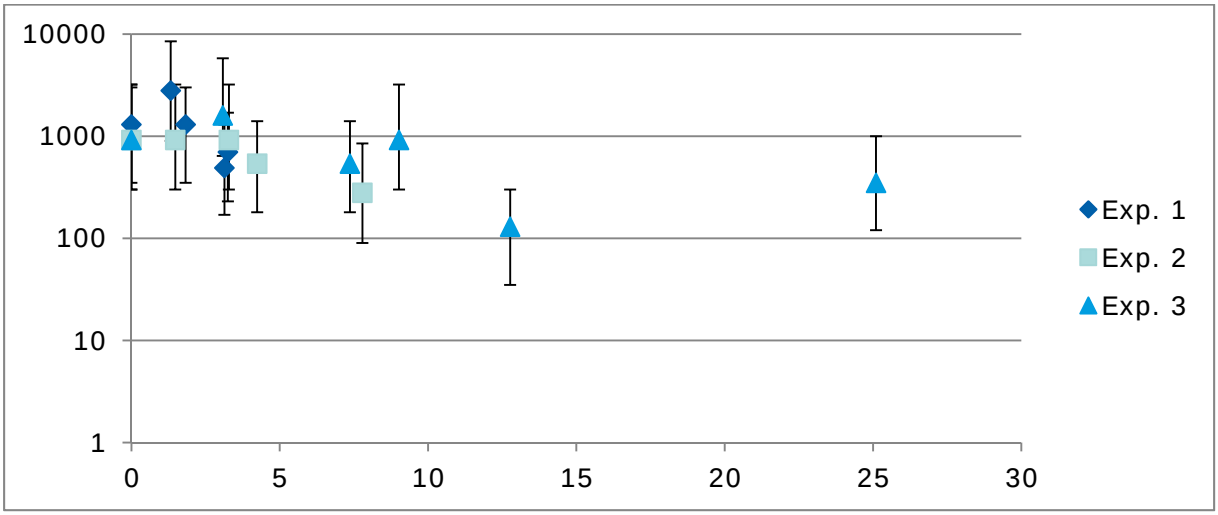

Figure 4. Concentration of amoebae [cells/mL] with 95% confidence intervals in error bars as function of ozone dose [mg Cl_2*min/L] for three experiments.

3.5 PCR confirmation

Because the culture used was not a clonal culture from a single amoebae cell but an isolation of a poly culture of amoebae from the gills of an infected fish, there is a chance that the repeated culturing of the amoebae could select for a different amoebae species than the intended *P. perurans*. Thus, a control of each culture used in the experiments was done by analysis of the control sample by PCR. This confirmed that *P. perurans* was present and multiplied in the control samples for each of the experiments, see **Figure 5** for positive PCR amplification and correct melting peaks.

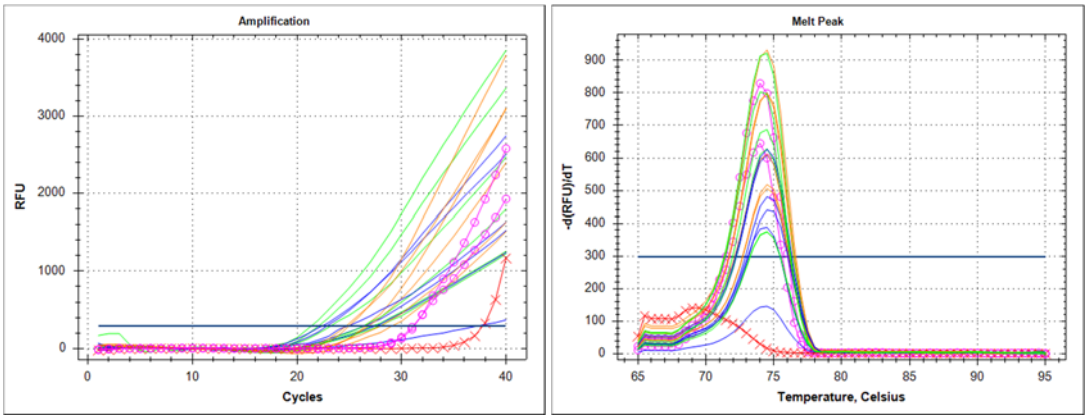

Figure 5: PCR amplification chart and melt peak for control samples targeting *P. perurans*. Each control sample is represented by a line in duplicate. The pink lines with circles are the positive PCR controls, while the red line with crosses is the non-template negative control.

4. Discussion

Instead of using cultures in exponential growth phase, the amoebae were harvested after entering a floating stage which is suspected to be a response to either high amoebae densities or nutrient deficiency. This was done partly to avoid having to physically scrape the cells from the surface and/or using trypsin to detach them, but mostly because it is the naturally formed floating stages that is most likely to be in the water entering the aquaculture systems. It has not been investigated whether the floating stage is more or less susceptible to disinfections compared to the attached stage with active pseudopods.

The amoebae were highly susceptible to UV treatment, and especially from medium pressure UV lamps. The susceptibility is comparable to most gram negative bacterial fish pathogens (Liltved et al., 1995), however, the amoebae were not killed by the low UV doses, but entered a viable but not cultivable state (VBNC). To induce acute damage and possibly kill the amoeba by UV irradiance, the doses must be in the same range as for AHN and IPN virus (Liltved et al., 2006). Apparently, medium pressure UV lamps needs lower doses for the same effect compared to low pressure UV lamps. This is because the doses are calculated based on the UV intensity at 254nm, while medium pressure UV lamps submits irradiation at a broad spectrum of wave lengths, and some of these wave lengths, or the combination of several wave lengths, are more potent than the single 254nm wave length from the low pressure UV lamps.

The VBNC state of the amoebae needs to be further investigated to see if they are only unable to multiply in the lab conditions, or if they are also unable to infect fish. A follow-up study will be performed at Solbergstrand with an infection study using UV-irradiated amoebae in the VBNC state.

Ozonated seawater did not have any effect on the amoebae in the concentrations tested in this experiment, even if the doses would inactivate a range of other fish pathogens (Itoh et al., 1997; Liltved et al., 1995; Liltved et al., 2006). The intention of the experiments was to repeat the same doses to show that the results were reproducible. However, the doses were increased for each experiment to try to find a dose that would give a significant effect, without success. A further increase in ozone doses seemed not worthwhile, as such high doses would be unrealistic to use in most practical industry-applications.

The experiment included indirect ozonation, meaning that the amoebae were not in direct contact with the ozone, but only a dilution of ozonated water. This design was chosen to represent an ozonation system where a side stream of the influent water is led to the ozone generator and then mixed with the main stream afterwards, or ozonation of a well boat where the water is circulated through an ozone generator. In both of the cases, ozone reacts with only a small part of the seawater, forms bromate and other oxidants, and it is these oxidants that are mixed with the main water body.

5. References

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