VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

Prospect of Hydroxyl Radical Exposure during Seawater Bathing to Treat Amoebic Gill Disease in Atlantic Salmon

This is the Published version of the following publication

The publisher's official version can be found at
http://dx.doi.org/10.1155/2023/5074580
Note that access to this version may require subscription.

WILEY | Hindawi

Research Article

Prospect of Hydroxyl Radical Exposure during Seawater Bathing to Treat Amoebic Gill Disease in Atlantic Salmon

Parvez Mahbub [1] and Thomas Yeager[1,2]

[1]*Institute for Sustainable Industries and Liveable Cities (ISILC), Victoria University, Footscray Park Campus, Melbourne 3011, Australia*
[2]*First Year College, Victoria University, Footscray Park Campus, Melbourne 3011, Australia*

Correspondence should be addressed to Parvez Mahbub; parvez.mahbub@vu.edu.au

This study aims to undertake hydroxyl ($^{\cdot}$OH) radical-based preliminary investigations with a view to utilising seawater as a viable alternative to freshwater for the treatment of amoebic gill disease (AGD) in aquaculture industries. The study presents *in vitro* viability studies of clonal amoebae species to examine the effects of $^{\cdot}$OH radicals on both parasites and hosts. The study also assesses the toxicity to Chinook salmon cell lines (CHSE-214) in freshwater and 35 ppt seawater via continuous dosing of 35 mM $^{\cdot}$OH radicals and hydrogen peroxide (H_2O_2) for 1.5 to 4 hr, separately at 18°C and 15°C. Comparatively high viability of CHSE-214 (60% in $^{\cdot}$OH and 50% in H_2O_2) for a prolonged treatment of up to 4 hr in seawater at 15°C indicates suitability of low seawater temperature while using either $^{\cdot}$OH or H_2O_2 during bathing. The viability of CHSE-214 remained relatively stable in seawater (55%–60% in $^{\cdot}$OH and 50%–60% in H_2O_2), at both temperatures of 18°C and 15°C. However, at 15°C, a drastic reduction of viability of CHSE-214 in freshwater (from 80% to 48% in $^{\cdot}$OH and from 70% to 58% in H_2O_2) has indicated high variations in toxicity levels in freshwater at low temperature. Using DNA staining agents in flow cytometry, the *in vitro* viability study results in >22.5% mortality of clonal *Neoparamoeba perurans* (NP), an AGD causative agent, in 35 ppt seawater containing 35 mM $^{\cdot}$OH radicals via one-off dosing for 1 hour at 15°C. In addition, fast radical consumption is more pronounced in the case of $^{\cdot}$OH radicals as compared to H_2O_2 in both freshwater and seawater due to extreme reactivity of the former. Hence, this study suggests that $^{\cdot}$OH radicals are detrimental to the viability of NP in seawater, and thereby, establishes grounds for further *in vivo* investigations of using seawater supplemented with continuous dosing of $^{\cdot}$OH radicals for Atlantic salmon bathing as a treatment of AGD.

1. Introduction

Atlantic salmon is a large international aquaculture industry valued at $13.7 billion US dollars annually. In 2015, the annual economic loss reported for treatment costs related to amoebic gill disease (AGD) in Atlantic salmon primarily caused by *Neoparamoeba perurans* (NP) ranged between 5 and 81 million US dollars for aquaculture farms in Tasmania, Ireland, Norway, and Scotland [1]. More than 11 amoebozoans within 6 amoebae genera, namely, *Neoparamoeba, Paramoeba, Vexillifera, Pseudoparamoeba, Vannella,* and *Nolandella* were classified as prevalent in AGD infected gills of Atlantic salmon by English et al. [2], although the

correlations between AGD and other amoebae species except for NP are not fully understood yet. Freshwater bathing of Atlantic salmon for 2–4 hr is the current recommended treatment for AGD [3]. The recurrence of AGD after initial bathing and the presumed stress on the fish resulting from the long bathing time of 2–3 hr are key challenges in freshwater bathing of Atlantic salmon. Additionally, collection of large volumes of freshwater from rivers and lakes up to 120 ML/month [4] may place pressure on freshwater resources. To date, alternative bathing approaches to treat AGD have been investigated including oxidisers, such as hydrogen peroxide (H_2O_2) [5], peracetic acid (PAA) [6], and sodium percarbonate (SPC) [7]. Preliminary studies of H_2O_2

and PAA have shown positive prospects for freshwater bathing of Atlantic salmon, whilst SPC did not demonstrate any positive outcome. Adams et al. [8] investigated various concentrations of H_2O_2 (500, 1000, and 1500 ppm) along with different bathing durations (10, 20, 30, and 60 minutes) in seawater for AGD treatment of Atlantic salmon. Whilst these studies have reported on bathing efficacies resulting from different dosing concentrations of oxidisers, the nature of dosing, in terms of one-off or continuous dosing of oxidisers, has not been explicitly investigated. In most cases, a one-off dosing at the start of the bath has been used as a benchmark for reporting bathing efficacies resulting from various concentrations of oxidisers. However, oxidisers decompose in water resulting in attenuation of the initial dosing concentration during the progression of a bath. An alternative approach would be a continuous dosing scheme that ensures a relatively stable concentration of oxidisers during the AGD bathing of Atlantic salmon. In this context, the continuous dosing of potent radicals such as hydroxyl (˙OH) radicals is quite challenging compared to other oxidisers, due to limited capacity for scalable production and lack of data regarding its efficacy in deactivation of NP. Recently, Mahbub et al. [9] demonstrated a portable and inexpensive technique capable of scalable production (between 40–90 mM) of ˙OH radicals from relatively low concentrations of H_2O_2 (between 0.1–5 M) in aqueous solution using a single continuous flow photoreactor. The direct use of seawater with the ˙OH radicals or H_2O_2 in aqueous solutions is an attractive approach which has the potential to reduce the use of natural freshwater from rivers and lakes for AGD treatment, depending on their toxicity to NP and Atlantic salmon. Recently, the authors in [10] observed that *in vitro* cold temperature (as low as 3˚C) acts as a stressor to NP by increasing their detachment from gills irrespective of the seawater or freshwater media. An important fact in this regard is that the average surface seawater temperature (SST) in southern Tasmania varies between 13˚ and 16˚C [11]. Therefore, *in vitro* addition of ˙OH radicals or H_2O_2 in aqueous seawater solutions at an average SST range is expected to enhance seawater's capability to act as an effective stressor to NP.

Flow cytometry studies of amoebae species have previously been reported. For example, Collins et al. [12] have sorted *Neoparamoeba perurans* (syn. *Paramoeba*) by cell size in clonal cultures to separate amoebae from the heterogeneous microorganism population using natural red (NR) staining and flow cytometry. The viability of *Acanthamoeba* spp. was reported by Borazjani et al. [13] using propidium iodide (PI) for staining the cytometric samples. Mi-Ichi et al. [14] employed calcofluor (CF) and Evans blue (EB) stains in a flow cytometric method to study the percentage of cysts during encystation caused by *Entamoeba histolytica* during an amoebiasis infection in humans. In this context, this study presents flow cytometry-based viability studies of NP and salmon cell lines subjected to ˙OH radicals and H_2O_2 prepared in freshwater or seawater solutions to provide a preliminary analysis of the toxicity of these oxidisers and potential as alternative treatments for AGD in Atlantic salmon.

2. Materials and Methods

2.1. Cell Line Culture. Chinook salmon embryo cell lines (CHSE-214) were obtained from CellBank Australia (91041114-CHSE-214-salmon embryo) and cultured according to company protocols (https://www.cellbankaustralia.com/chse-214.html). CHSE-214 cell lines were cultured in Eppendorf® T75 cell culture flasks (Eppendorf, Germany) with Gibco™ minimal essential medium (MEM) with L-glutamine (11095080), 1% Gibco™ MEM nonessential amino acids solution (100X) (11140050), and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) in a New Brunswick Galaxy® 170 R CO_2 incubator. The primary reason for using CHSE-214 cell lines was their easy availability and quick culture protocol as well as use of this cell line by other researchers during their investigations of loss of virulence of clonal NP [15].

2.2. Wild Amoebae Including NP Collection and Transfer from Gills. The gill basket of six farmed Tasmanian Atlantic salmon (Huon Aquaculture Pty Ltd) that displayed clinical signs of AGD with a gill index greater than 3 [16] were dissected, placed in 50 mL falcon tubes containing seawater, and transported to Victoria University, Melbourne, on the same day of collection at 2–4˚C. Seawater was collected from the same sea cages for amoebae culture media preparation in the laboratory. The pH and salinity of the seawater during collection were recorded as 8 and 35 ppt, respectively. Gills were transferred to T75 culture flasks in 25 mL seawater and placed on an orbital shaker for 60 min. Gill mucus was scraped off the gill surface with a sterile scraper and collected in the T75 flask. The remaining gill tissue was discarded. NPs were allowed to attach to the culture flask by simply leaving the flask still for 15–20 min. The seawater, floating mucus, and other gill debris were discarded and replaced with 18 mL fresh 35 ppt seawater. The gill transport seawater in 50 mL falcon tubes was centrifuged at $3000 \times g$ for 5 minutes to collect NP dislodged during transport. The supernatant was discarded and pellets were resuspended in 2 mL 35 ppt seawater and added to the previous T75 flask. Finally, 200 μL 10X MYB (1 g malt, 1 g yeast, 1 L sterile seawater) was added to the T75 flask and incubated at 14˚C in a 23 L Kogan® thermoelectric fridge. Media were changed every five days, and NP passaged every three weeks by centrifugation at $3000 \times g$ for 5 min. Experiments were undertaken on flasks when NP confluence reached 80%. Two days before the experiments, T75 flasks were subjected to 10X penicillin/streptomycin (Sigma Aldrich, Australia) to reduce any bacterial load in seawater. In order to investigate the impact of different (˙OH) radical concentrations on the DNA of wild NP, this study undertook quantitative PCR (qPCR) by utilising a universal primer (based on 18S ribosomal RNA, please refer to qPCR methods and Table S1 in supplementary information) for eukaryotes, especially amoeba in the sample. Due to the presence of different amoebozoans in AGD infected gills along with wild NP as mentioned earlier in introduction, this study does not claim that the qPCR results specifically represented wild NP DNAs exposed to

various (·OH) radicals. Nonetheless, as the aim of this study was to investigate the prospect of using seawater as a generalised remedy of AGD targeting a wide range of wild amoebae that could be present in the salmon gills, the qPCR methods and results are presented in supplementary information for interested readers. In order to maintain the specificity of the impact of (·OH) radicals on NP, clonal NP cell lines were employed in the subsequent investigations.

2.3. Clonal Neoparamoeba perurans (NP) Cell Line. A clonal NP cell line cultured in seawater was provided by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) [17]. Falcon tubes containing the clonal NP were gently shaken before transfer of 25 mL to two T75 culture flasks. The media were converted to 1X MYB with the addition of 200 μL 10X MYB (1 g malt, 1 g yeast, and 1 L sterile seawater) to the T75 flask. NP was incubated at 14°C in a 23 L Kogan® thermoelectric fridge. Media were changed every five days, and NP passaged every three weeks by centrifugation at $3000 \times g$ for 5 min. Experiments were undertaken when the NP flasks reached 80% confluence. Two days before experiments, T75 flasks were subjected to 10X penicillin/streptomycin as previously described.

2.4. Toxicity of Hydroxyl Radicals and Hydrogen Peroxide to Cell Lines. As Huon aquaculture currently employs freshwater sourced from nearby rivers for bathing of Atlantic salmon, a safe maximum limit for exposure of CHSE-214 cell lines at various concentrations of aqueous H_2O_2 was assessed by replacing growth media with H_2O_2 in 20 mL of raw freshwater sourced from the Huon River in Tasmania for a maximum of 4 hours at 18°C (more discussions of the safe maximum limit are provided later in Section 3.1). The use of freshwater also ensured maximum exposure of CHSE-214 cell lines to H_2O_2, as the bacterial load is generally expected to be lower in freshwater than that in seawater [18]. CHSE-214 cells were 80% confluent with a density of approximately 716,000 cell/mL as determined by counting using a hemocytometer. CHSE-214 cell lines were observed by microscopy at 100× magnification using a Kern microscope camera (ODC832, Kern and Sohn GMBH, Germany). CHSE-214 cell lines were then also exposed to ·OH radicals from 1 to 50 mM produced from the parent material of 50 mM H_2O_2 in aqueous solutions using a custom built continuous flow photoreactor as previously described by Mahbub et al. [9]. Wild amoebae extracted from gills were exposed to 10 mM–50 mM ·OH radicals for 1 hr as detailed in Table 1. These amoebae were cultured in 35 ppt seawater in a similar manner as the clonal NP as described in the previous section. After 1 hr of treatment in various concentrations of ·OH radicals, we removed the media with fresh seawater. So, there were no residual ·OH radicals left in the culture media. Cells were counted using a hemocytometer and transferred to a 24-well plate (SIAL 0524, Sigma®cell culture plate) at ~20,000 cells/mL. Two days before the treatment with ·OH radicals, wells were treated with 10X penicillin/streptomycin as previously described.

2.5. Adherent NP and CHSE-214 Hydroxyl Radical and Hydrogen Peroxide Studies. After gently shaking for 5 min, confluent clonal NP was transferred by pouring to T75 flasks containing 80% confluent CHSE-214 cell lines for experiments after discarding the media. NP was visually observed by microscopy for settlement on the CHSE-214 cell lines. Hydroxyl (·OH) radicals and H_2O_2 exposure experiments were started immediately on settlement. Culture media were replaced with either 5 mL of seawater or freshwater and 11.9 mL of 50 mM ·OH radicals or 11.9 mL and 50 mM 30% (w/w) H_2O_2 (Sigma Aldrich 216763) added for a final concentration of 35 mM at the start of bathing. Experiments were carried out at controlled ambient temperature of 15°C and 18°C inside a CO_2 incubator (New Brunswick Galaxy® 170 R). A 1.5 mL sample was collected from each flask at the start of bathing to measure residual H_2O_2 in each flask. Culture flasks were continuously dosed with 200 μL/min of either 35 mM ·OH or 35 mM H_2O_2, and 1.5 mL samples were collected at 1 hr, 1.5 hr, 2 hr, and 4 hr to check H_2O_2 and ·OH radical levels. Culture flasks were visually observed by microscopy at 100× magnification using a Kern microscope camera (ODC832, Kern and Sohn GMBH, Germany) at each time point.

At the end of the 4 hr bath, the media were poured into 50 mL falcon tubes and 2 mL 0.25% trypsin was added to each T75 culture flasks. After shaking for 5 min, culture flasks were supplemented with 5 mL of MEM enriched with 10 X nonessential amino acids, 10X FBS, and 50X penicillin/ streptomycin. Detached cells were collected in 10 mL Falcon tubes by centrifugation at $500 \times g$ for 5 min. The supernatant was then discarded, and cells were resuspended in 2 mL of either freshwater or seawater.

2.6. Flow Cytometry. Flow cytometry was undertaken to measure the percentage viability of CSIRO clonal NP cells after treatment with ·OH radicals. Three replicate cytometer tubes representing each experiment were prepared following the similar protocol described as follows. Culture flasks containing NP were pretreated with 10X penicillin/strep-tomycin as previously described. The concentration of NP was measured using a hemocytometer for a final concentration of 100,000–300,000 cells/mL in the T75 flasks. Each experiment was accompanied with a "control" flask, which was not subject to any treatment, but the media were replaced with an equal volume of either 0.25 μm filtered seawater or freshwater at the start of the treatment at corresponding temperatures. NP was detached from culture flasks by gently shaking the flask for 5 min, ensuring all NPs were resuspended in seawater. NP was centrifuged at $3000 \times g$ for 5 min, the supernatant was discarded, NP was resuspended in 2 mL seawater, and 0.3 mL were transferred to 5 mL cytometry tubes. The NP was then treated with 35 mM ·OH solution prepared in situ using our photoreactor by dispensing 0.7 mL ·OH radicals @100 μL/min for 7 min and then left for 60 min treatment. The NP sample was then injected with 10 μL SYBR safe nucleic acid DNA staining agent (Thermo Fischer Scientific S33102) (excitation at 280 nm and 510 nm and emission at 530 nm) 5 min before

TABLE 1: Experimental conditions for CHSE-214, wild amoebae, and clonal NP.

Cell lines or amoebae	H_2O_2 conc. (mM)	·OH conc. (mM)	Treatment duration (min)	Media	Temperature (°C)	Further analysis	Remarks
	1	—	30	Freshwater (pH 6.5)	18	Microscopy	Initial experiment to establish a safe maximum limit of *in vitro* H_2O_2 concentration towards CHSE-214 as a parent reagent
	50	—	60				
	100	—	240				
		—	5				
			90	Freshwater (pH 6.5)	18		
CHSE-214	35	35	120	Freshwater (pH 6.5)	15	Flow cytometry	Comparing *in vitro* viabilities of salmon cell lines exposed to safe H_2O_2 and ·OH concentrations at different durations, media, and temperatures via continuous dosing
			120	Seawater (pH 8, 35 ppt)	18		
			240	Seawater (pH 8, 35 ppt)	15		
		35	60	Seawater (pH 8, 35 ppt)	15	Flow cytometry	Validating previous experiment in seawater at 15°C but shorter treatment duration to check high *in vitro* viability of salmon cell lines via continuous dosing
Clonal NP	—	35	60	Seawater (pH 8, 35 ppt)	15	Flow cytometry	Validating *in vitro* lethal dose for clonal NP in seawater at 15°C via one-off dosing
Wild amoebae adhering to CHSE-214	—	35	60	Seawater (pH 8, 35 ppt)	15	Microscopy	Visual check of *in vitro* lethal dose to wild amoebae in seawater at 15°C via one-off dosing
Clonal NP adhering to CHSE-214	—	35	60	Seawater (pH 8, 35 ppt)	15	Microscopy	Visual check of *in vitro* lethal dose to clonal NP in seawater at 15°C via one-off dosing

the injection of samples into a BD AccuriTM C6 Plus flow cytometer with a fluid flow rate of $10 \mu L/min$ and a core size of $10 \mu m$. A control sample tube without treatment was also included. Flow cytometry data were analysed using the BD AccuriTM C6 Plus built-in software package. Polygon gating and histogram functions of cytometry software were utilised to interpret the cytometry data.

Flow cytometry was also undertaken to measure the toxicity towards CHSE-214 cells through continuous dosing of 35 mM ($595 mgL^{-1}$) $\cdot$OH radicals and 35 mM ($1191 mgL^{-1}$) H_2O_2 in both seawater and freshwater at $15°C$ and $18°C$ for 1.5 to 4 h as previously described. Propidium iodide (PI) (Thermo Fisher Scientific P1304 MP) was used to differentiate live and dead populations of CHSE-214 cells in four different treatment conditions consisting of freshwater at $15°C$ (FW15°C), freshwater at $18°C$ (FW18°C), seawater at $15°C$ (SW15°C), and seawater at $18°C$ (SW18°C) for each treatment group. PI is a membrane-impermeable fluorescent DNA stain. It is used to stain apoptotic cells for detection via flow cytometry [19]. Samples were injected with $10 \mu L$ PI (excitation at 540 nm and emission at 630 nm) for each 1 mL sample from each experiment and allowed 5 min to stain nuclear DNA of the salmon cells. Samples were injected onto a BD AccuriTM C6 Plus flow cytometer with a fluid flow rate of $10 \mu L/min$ and a core size of $10 \mu m$. A control sample without treatment was also included. Flow cytometry data were analysed using the BD AccuriTM C6 Plus built-in software package. Viabilities of both clonal NP and CHSE-214 at 35 mM $\cdot$OH radicals in seawater for 1 hr through flow cytometry were investigated on two separate days in order to determine the repeatability of the in vitro treatment process. Results of these repeat experiments are given in the supplementary information.

2.7. Ethics Statement. The "Tissue Notification Form" for this project was submitted and approved by the Victoria University Animal Ethics Committee (aeec@vu.edu.au) on 22 July, 2020 (AEC Tissue Notification Form, Revised version 4/2/2016).

3. Results and Discussions

3.1. Safe Limit of Hydrogen Peroxide as a Parent Reagent. Caution needs to be exercised in extrapolating in vitro toxicity of H_2O_2, particularly due to the fact that a lethal in vitro dose to amoebae may not be safe for CHSE-214 [5]. Therefore, initially, a safe maximum limit of parent material (i.e., H_2O_2) was established in aqueous solution for CHSE-214 cell lines for a maximum 4 hr bathing time by replacing the growth media within 1 mM–100 mM concentration ranges of H_2O_2 in freshwater in the T75 culture flasks (as described in methods and Table 1), and we observed the CHSE-214 cells by microscopy (Figure 1). This initial concentration range was chosen to include concentrations of H_2O_2 investigated in previous studies (e.g., [8]) of AGD treatment.

Figures 1(a)–1(c) demonstrate the exposure of the CHSE-214 cell lines to a low concentration of H_2O_2 (1 mM or 34 ppm) in the T75 culture flasks by replacing growth media with freshwater. No visible sign of distress was observed to the salmon cells during the treatment for up to 60 min by microscopy (Figures 1(a)–1(c)). Exposure of CHSE-214 cells to 50 mM H_2O_2 for 4 hours was harmless (Figure 1(d)); however, 100 mM H_2O_2 resulted in extensive damage and detachment of the cell lines from the bottom of the flask which is visible from the beginning of the exposure period (Figure 1(e)). Nowak et al. [20] have previously advised caution when extrapolating in vitro to in vivo results in their study of in vitro exposure of 3000 NP cells/mL to 1000 ppm or 29.4 mM H_2O_2 for 10–20 minutes in seawater which resulted in 0–20% survival rates at the end of treatment; however, in vivo exposure of AGD-infected salmons to 1250 ppm or 36.8 mM H_2O_2 at 12 and 18°C for 15 minutes indicated osmoregulatory stress in salmons. Such caution also applies to the in vitro scale. Other in vivo experiments have shown that Atlantic salmon exposed to H_2O_2 as high as 1250 ppm at 16°C do not affect aerobic performance of salmon and have a negligible impact on their hypoxia tolerance [21]. The high degree of variability in the efficacy of H_2O_2 bathing may be attributed to the variable reactivity of H_2O_2 in the presence of divalent metal cations, catalysts, exposure to direct sunlight as well as presence of inhibitors in the aqueous solutions [22]. Reactivity of H_2O_2 is quite slow at moderate temperatures without the presence of any catalyst [23]. To the contrary, reactivity of H_2O_2 speeds up at increased temperature, which triggers the thermal decomposition of H_2O_2. In contrast, hydroxyl radicals ($\cdot$OH) are extremely reactive irrespective of temperature (1.6× more than H_2O_2 based on standard reduction potentials; [24]). Therefore, in our subsequent in vitro investigation of wild amoebae as well as clonal NP presented in the following sections, 50 mM H_2O_2 was employed as the parent material converted into various concentrations of hydroxyl radicals (10 mM to 50 mM $\cdot$OH radicals) using our scalable photoreactor described elsewhere [9].

3.2. Toxicity Results on Clonal Neoparamoeba perurans Adhering to CHSE-214 Cells. Our study investigated the effect of 35 mM (595 ppm) $\cdot$OH radical on the clonal NP supplied by CSIRO adhering to the CHSE-214 salmon cells in 24-well culture plates. Based on the in vitro toxicity data for wild amoebae from salmon gills (provided as supplementary information in sections S2 and S3.1), 35 mM (595 ppm) $\cdot$OH radicals were applied as a conservative approach in all of our subsequent experiments. Figure 2 shows clonal NP cells adhering to CHSE-214 cells before and after treatment with 35 mM $\cdot$OH radicals in seawater. The room temperature during the treatment was 17°C, and the seawater temperature was 15°C. The transfer protocol of clonal NP cells allowing them to adhere to the CHSE-214 cell lines is provided in the ESI.

A previous in vitro study by Cano et al. [25] using sophisticated polyester membrane-based transfer wells showed adherence of NP to rainbow trout gill cell lines in seawater in 20 min. In our investigation, it was also observed that the NP cells adhered to the CHSE-214 within 5 min of transfer (Figure 2) using a less sophisticated cohabitation
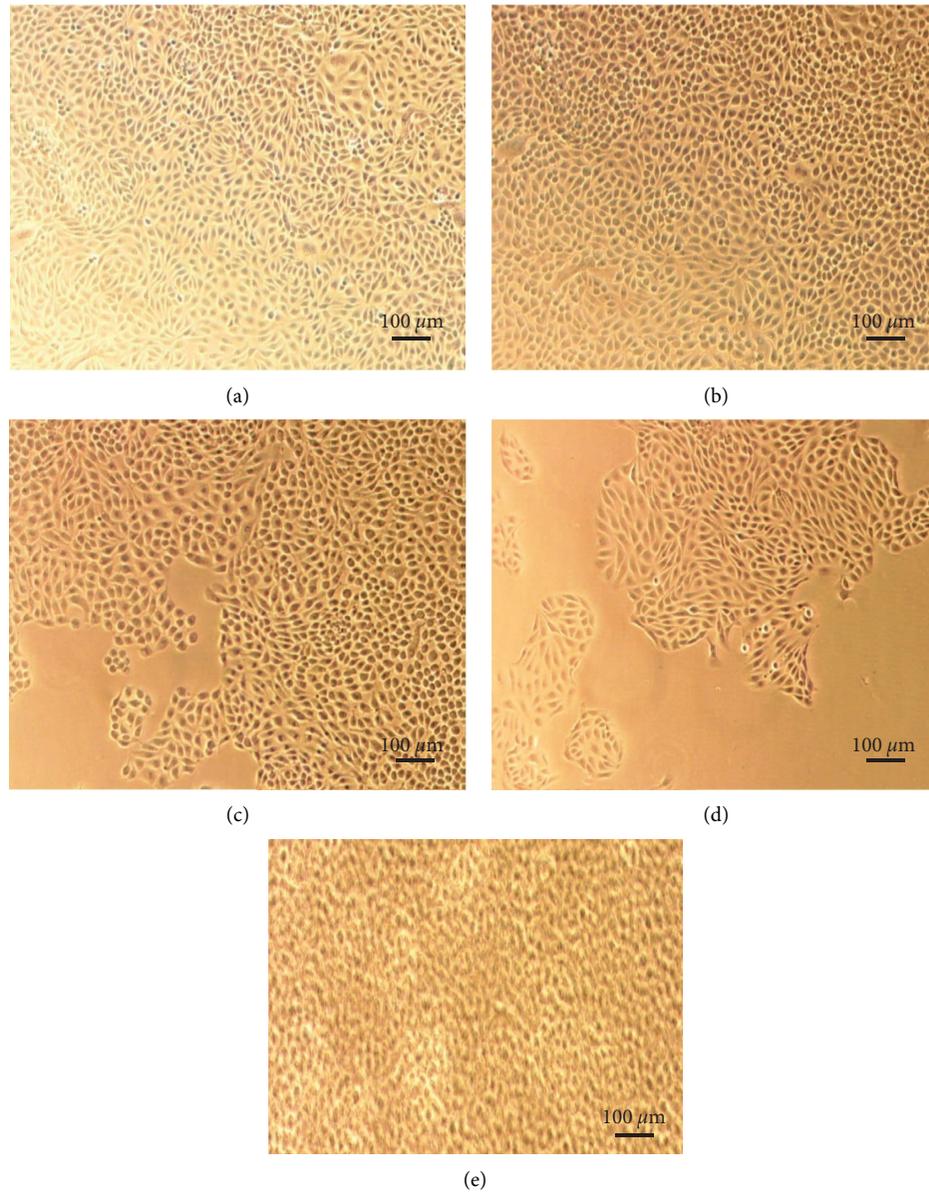
(a)

(b)

(c)

(d)

(e)

FIGURE 1: Exposure of CHSE-214 cells to hydrogen peroxide (H_2O_2) in freshwater: (a) preexposure; (b) 30 min at 1 mM (34 ppm) H_2O_2; (c) 60 min at 1 mM (34 ppm) H_2O_2; (d) 240 min at 50 mM (1700 ppm) H_2O_2; (e) damaged cells at the beginning of the 100 mM (3400 ppm) H_2O_2 bath (magnification: 100X); a change in focal plane in Figure 1(e) also suggests that cells might be detached from the bottom surface of the flask.

experimentation method. NP cells formed a cyst-like morphology after application of 35 mM (595 ppm) ·OH radicals for 60 min. However, the CHSE-214 salmon cells remained visibly unharmed at the end of 60 min (Figure 2). The density of live CHSE-214 cell lines before treatment was 760,000–1,000,000 cells/mL (in Figure 2(a)) as determined by counting via a hemocytometer from three replicate culture flasks, which remained approximately 700,000–960,000 cells/mL (in Figure 2(b)) after 1 hr of treatment in 35 mM ·OH radicals in seawater. To the contrary, the count of live clonal NP before treatment was ~53,000 cells/mL (in Figure 2(a)), whilst viability reduced to ~40,000 cells/mL in Figure 2(b) (considering the cyst-like morphology of clonal NP after treatment was still viable).

This roughly accounts for ~25% of the clonal NP as dead after the 1 hr treatment in 35 mM ·OH radicals in seawater at 15°C.

3.3. Viability of Neoparamoeba perurans Assessed by Flow Cytometry. The fate of clonal NP after a one-off dose of 35 mM ·OH radicals in seawater for 60 min at 15°C is presented in this section. Figure 3 illustrates the cytometer outputs in the control sample.

Initially, in the NP sample tube of the cytometer, a significant presence of live NP cells was observed (Figure 3(a)) which is confirmed by the fluorescence intensity plot (Figure 3(c)). There are some dots scattered around the
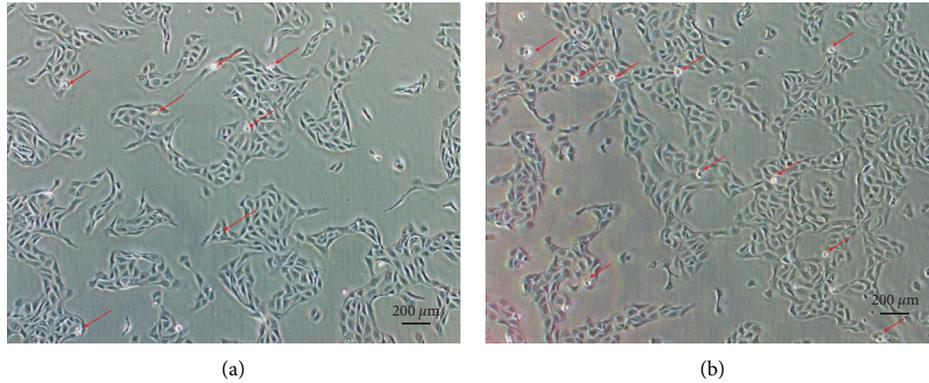
Figure 2: Clonal NP cells adhering to Chinook Salmon Embryo (CHSE)-214 cells treated with 35 mM (595 ppm) hydroxyl radicals (a) pretreatment (5 minutes after transfer) and (b) 60 minutes of treatment. Magnification: 100X.
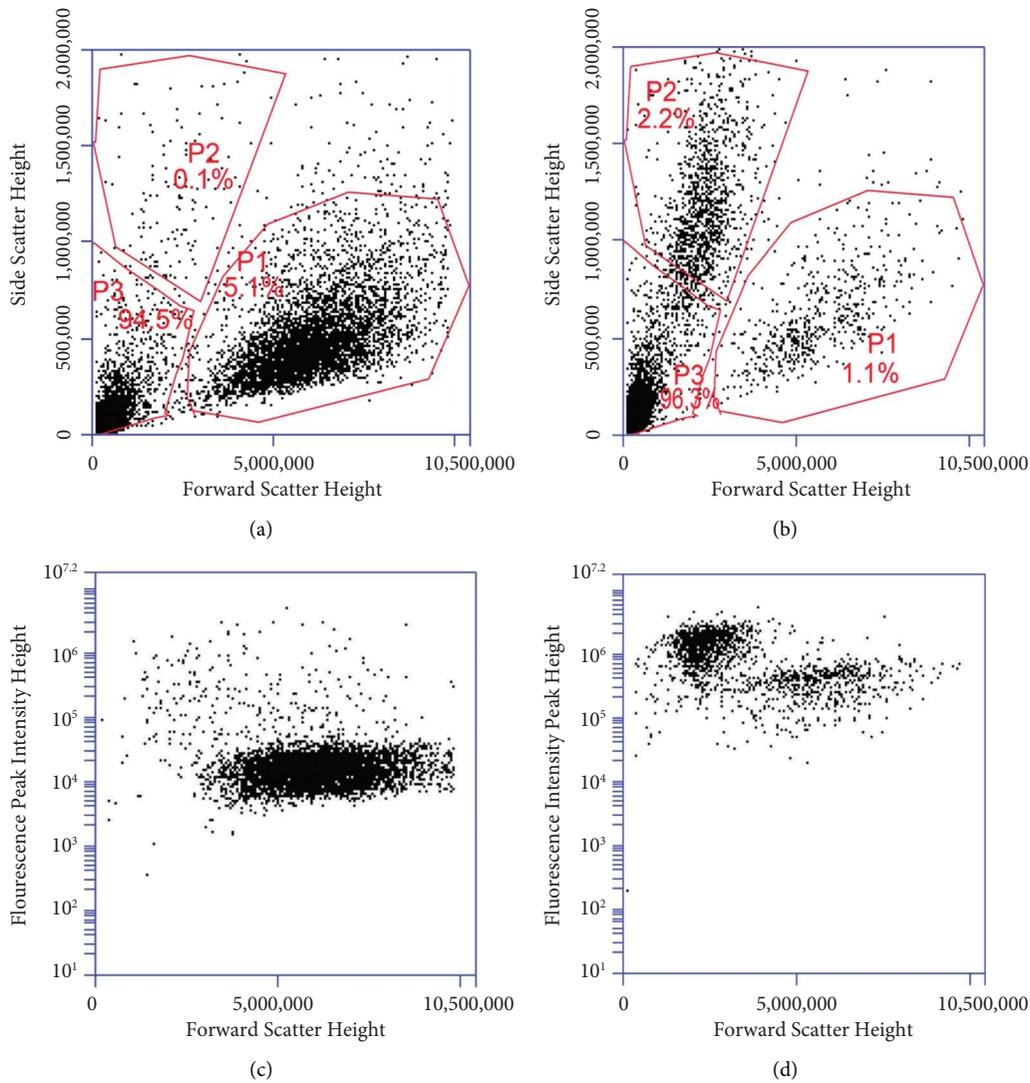

Figure 3: (a) Live NP cells in the cytometer tube are illustrated within the red polygon (P1) in terms of forward scatter height and side scatter height of light scattered from NP cells in seawater. The other polygons contain the presumably dead NP cells (P2) and contaminants in fresh seawater (P3). (b) Changes in viability of NP after 60 min ˙OH radical treatment in the cytometer tube. (c) A significant cluster of live NP cells through gating of P1 and P2 against the whole population of A before the start of treatment. (d) Separate clusters of live and dead NP cells after 60 min of ˙OH radical treatment through gating of P1 and P2 against the whole population of B.

cluster in Figure 3(c) which is indicative of preexisting dead NP cells before the start of treatment. At the end of 60 min ·OH radical treatment, live NP population was observed to reduce to one-fifth of its initial percentage (i.e., 5.1% in P1 in Figure 3(a) reduced to 1.1% in P1 in Figure 3(b)), whilst dead NP population significantly increased in this period (i.e., 0.1% in P2 in Figure 3(a) increased to 2.2% in P2 in Figure 3(b)). Histograms also confirm a high count of live NP cells (Figure 4(a)) compared to dead NP cells (Figure 4(c)) in the cytometer tube before the start of 60 min ·OH radical treatment. This result is expected if all NP in a culture were transferred rather than only attached NP. To the contrary, a marked decrease was observed in the counts of live NP (5203 counts in Figure 4(a) to 578 counts in Figure 4(b)) and increase of dead NP (119 counts in Figure 4(c) to 1169 counts in Figure 4(d)) via histograms after the 60 min ·OH radical treatment. The important fact to be noted here is that only NP cells within the polygons P1 and P2 in Figures 3(a) and 3(b) were accounted via cytometry. However, there is a high chance that some dead NPs might fall outside P2 as well as overlapped with P3 as evident in Figure 3(d). Nonetheless, our accounting of NP cells reveals at least 22.5% of the live NP population is confirmed dead after the 60 min ·OH radical treatment.

Thus, our simple flow cytometry protocol with the SYBR safe DNA staining agent has contributed to our investigation to confirm the adverse effects of one-off dosing of 35 mM ·OH radicals for 60 min on NP cells resulting in–22.5% mortality of NP. It is naturally anticipated that these single-cell NP would be under more stress if the one-off dosing is replaced with a continuous dosing of 35 mM ·OH for different durations. As mentioned in the introduction, one-off dosing of other oxidisers such as H_2O_2, PAA, and SPC has already been investigated for AGD treatment by other researchers. Therefore, instead of repeating similar investigations involving one-off dosing of H_2O_2 on NP, the effects of continuous dosing of ·OH and H_2O_2 only on CHSE-214 at different temperatures and media were rather investigated in the next section, considering the natural fact that continuous dosing of ·OH radical to NP would cause much higher mortality than the one-off dosing of ·OH.

3.4. Viability of CHSE-214 Cells Recorded via Flow Cytometry.

CHSE-214 cell lines were subjected to continuous dosing of 35 mM ($595 \, mgL^{-1}$) ·OH radicals and 35 mM ($1191 \, mgL^{-1}$) H_2O_2 in both seawater and freshwater at 15°C and 18°C to assess toxicity. Figure 5 shows the viability statistics of CHSE-214 cells at four different treatment conditions consisting of freshwater at 15°C (FW15°C), freshwater at 18°C (FW18°C), seawater at 15°C (SW15°C), and seawater at 18°C (SW18°C) for each treatment group.

The control flask in seawater exhibited normal behaviour with the highest viability of $82 \pm 2.2\%$ and $68 \pm 2.9\%$ at 15°C and 18°C, respectively. In contrast, freshwater is likely to induce osmotic shock resulting in low viability (45%–60%) in the control flasks in comparison to the greater viability (68%–82%) observed in control flasks in seawater. It is also interesting to observe that the viability increased in seawater

from 68% to 83% with a decrease in temperature from 18°C to 15°C. This trend was opposite in freshwater where viability increased slightly from $\sim43 \pm 2.9\%$ to $62 \pm 2.3\%$ when temperature increased from 15°C to 18°C. While suitable pH and temperature favour the utilization of metals to enhance the viability of inhabitants in both seawater [26] and in freshwater environments [27], a markedly different metal distribution in these two ecosystems might affect the patterns/pathways towards viability. Although increased viability at decreased temperature can be generally expected, the relatively less abundance of divalent transition metal cations in freshwater may result in a different pathway towards the viability of its inhabitants. From our investigations of the viabilities of CHSE-214 in control flasks of Figure 5, it would be reasonably agreeable that, irrespective of treatment durations, the viabilities of CHSE-214 cell lines in control flasks were always higher in seawater compared to freshwater for the same temperature.

In the treatment flasks, irrespective of temperature differences, one common feature observed in both seawater and freshwater was that shorter treatment times always improved viability in both ·OH radical (1.5 hr: ~80% in FW 18°C; 2 hr: ~48% in FW 15°C) and H_2O_2 (1.5 hr: 68% in FW 18°C; 2 hr: 55% in FW 15°C) in freshwater as well as in both ·OH radical (2 hr: 60% in SW 18°C; 4 hr: 60% in SW 15°C) and H_2O_2 (2 hr: 65% in SW18°C; 4 hr:50% in SW15°C) in seawater. Therefore, treatment periods shorter than 4 hr are preferable when treating with either ·OH radicals or H_2O_2.

For the best-case scenario of the control flask (i.e., SW at 15°C and 4 hr treatment), ·OH radical treatment showed greater CHSE-214 viability (60%) compared to H_2O_2 (50%). The load of organic matter (including microorganisms) is very different in seawater and freshwater, with seawater potentially possessing larger concentrations of oxidisable organic load than freshwater. Hence, it is expected that highly reactive ·OH radicals will be more rapidly consumed via oxidation in seawater than in freshwater, leaving less amount of ·OH radical in seawater to attack the CHSE-214 cell line. Elevated pH and low temperature may be the two most important parameters to govern the rate of this oxidation. This is attributed to the relatively high viability of CHSE-214 cells treated with ·OH radicals to the fact that ·OH radicals are 1.6 times more reactive towards oxidisable matter present in the aqueous media compared to the H_2O_2 (standard reduction potential of ·OH = 2.8 eV as compared to that of H_2O_2 = 1.78 eV). Hence, at the same concentration of 35 mM, the "contribution/tendency" of ·OH radicals in deactivating the microorganisms present in either seawater or freshwater could have been much higher than that of H_2O_2 employed for the same purpose. In other words, a significantly higher amount of ·OH radicals compared to H_2O_2 will be expended in deactivating the microorganisms present in aqueous solutions. This may have resulted in more H_2O_2 present at the cell lines' interface compared to ·OH radicals, resulting in higher viability of cell lines in ·OH radical flasks. To verify this assumption, this study investigated the consumption rate of both ·OH and H_2O_2 in the T75 culture flasks containing the CHSE-214 cell lines during the four different treatment scenarios. Machine
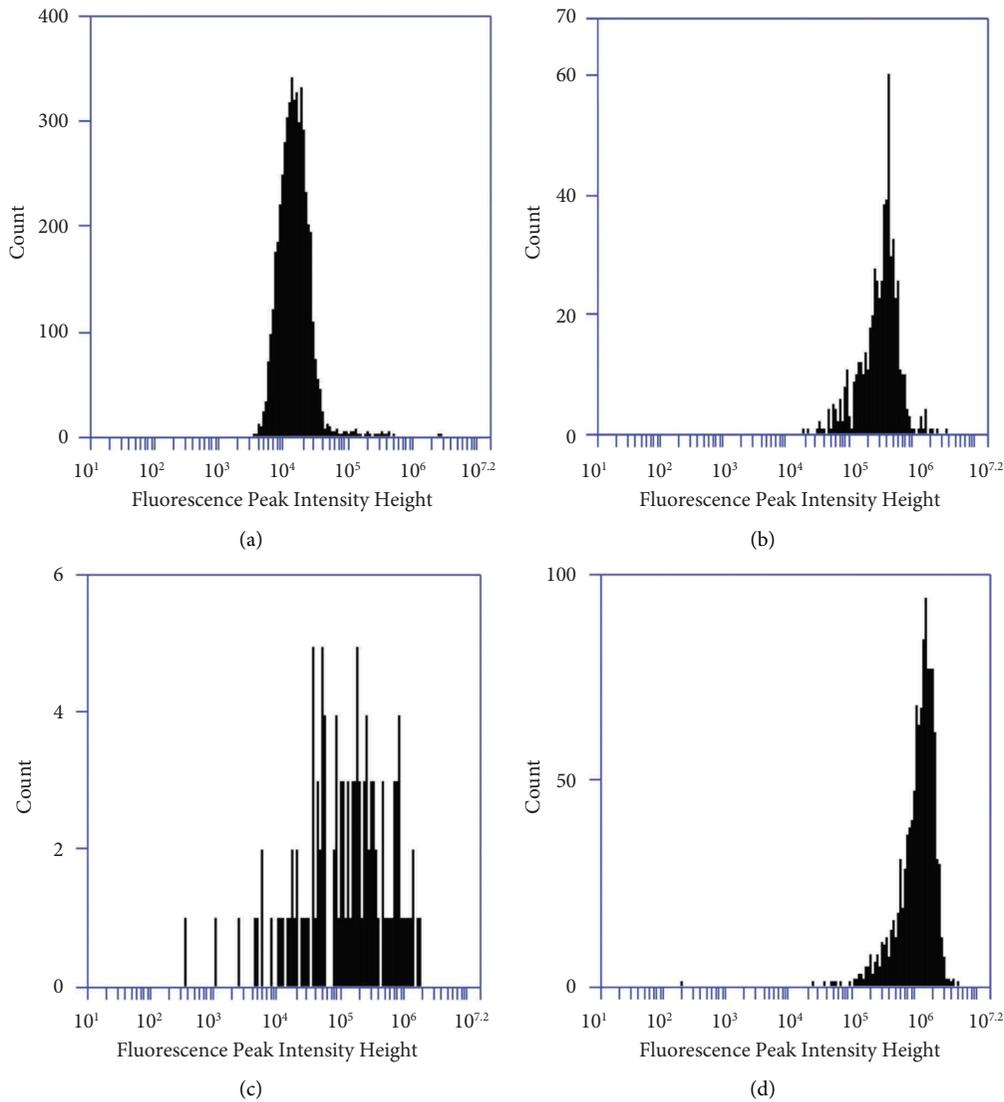
(a)

(b)

(c)

(d)

FIGURE 4: Histogram of live NP cells in terms of mean fluorescence intensity height count, (a) before treatment, and (b) after treatment. The histogram of dead NP cells in terms of mean fluorescence intensity height count, (c) before treatment, and (d) after treatment.

malfunction prevented recording of the ·OH and H_2O_2 consumption in seawater at 18°C (·OH SW18 and H_2O_2 SW18). Figure 6 shows the results for the remainder of the combinations of seawater (SW) and freshwater (FW) at both 18°C and 15°C.

As expected, from 10 min to 240 min, the consumption of ·OH radicals in the culture flask is 4–4.5 fold greater than that of H_2O_2 at a CHSE-214 cell confluence of ~80% irrespective of differences in temperature and media. Considering our previously selected best-case

scenario in Figure 6 (i.e., SW at 15°C and 4 hr treatment, both ·OH and H_2O_2), it is observed that, after 240 minutes from the start of treatment, about 45 mM H_2O_2 is present in the T75 culture flask compared to about only 5 mM ·OH radical, and this trend is evident for other treatment durations. Therefore, this high concentration of H_2O_2 remaining in the solution after the prolonged treatment period of 4 hr may have resulted in the low viability of CHSE-214 in seawater for SW at 15°C treatment using H_2O_2.
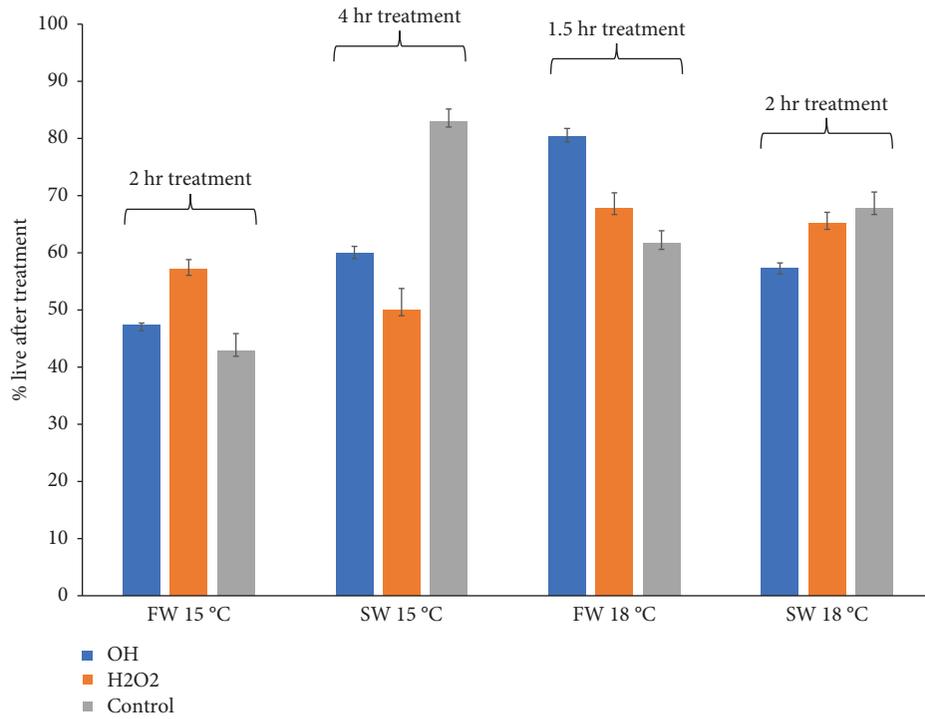
FIGURE 5: Percent viability of CHSE-214 cells after treatment with 35 mM hydrogen peroxide (H_2O_2) and hydroxyl ($^{\cdot}OH$) radicals in seawater or freshwater for 1.5 to 4 hr. Error bars represent the standard deviation of three replicate cytometer tubes for each experiment. Corresponding flow cytometry plots of each experimental condition are provided in Figures S8-S9 in supplementary information.
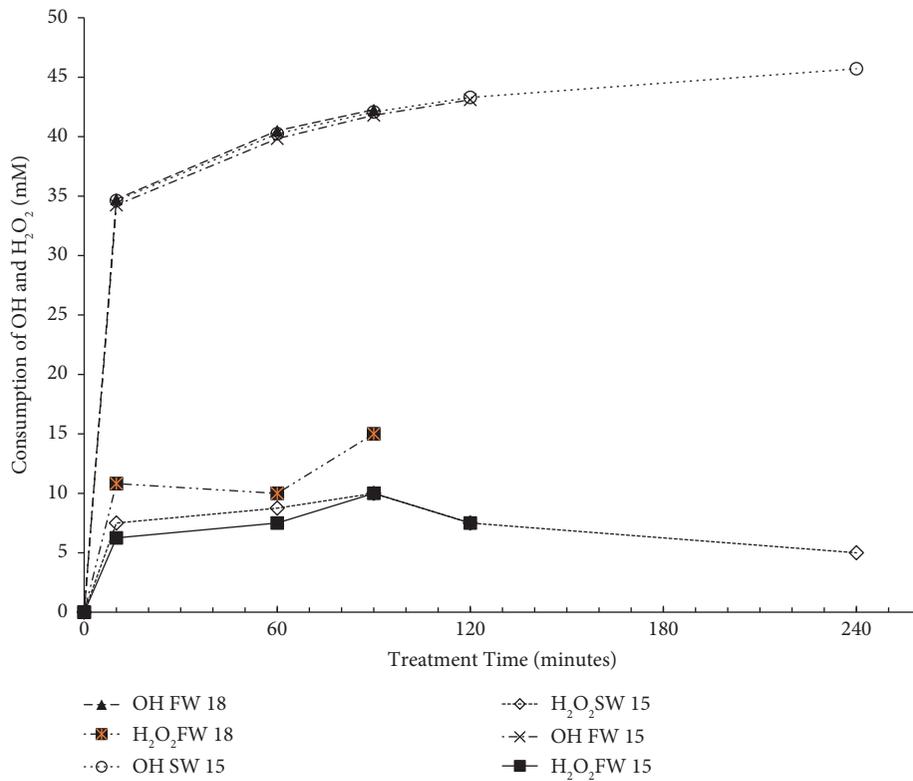

FIGURE 6: Radical consumption in T75 culture flasks containing CHSE-214 salmon cell lines contained in 20 mL fresh seawater or freshwater during continuous dosing for various treatment times. The starting reagent is 50 mM H_2O_2 dosed continuously at 100 μL/min whilst 50 mM H_2O_2 was converted into 50 mM $^{\cdot}OH$ radical using the photoreactor and then dosed continuously.

4. Conclusions

This study has established preliminary grounds for implementing continuous dosing of ˙OH radicals in seawater for AGD treatment. The *in vitro* investigations showed that seawater supplemented with ˙OH radicals at 15°C to 18°C has the potential to be a sustainable alternative to freshwater bathing of Atlantic salmon for the treatment of AGD. Flow cytometric studies of clonal NP showed significant mortality (>22.5%) on exposure to a single one-off dosing with 35 mM ˙OH radicals for 1 hour at 15°C in 35 ppt seawater. At 15°C in 35 ppt seawater, CHSE-214 cell lines demonstrated increased viability (up to 82%) during the continuous dosing of 35 mM ˙OH radicals compared to 35 mM H_2O_2. This *in vitro* study also demonstrated higher reactivity of ˙OH in both seawater and freshwater towards clonal NP and CHSE-214 compared to the reactivity of H_2O_2. Therefore, 35 mM ˙OH radicals can be implemented in large-scale studies of continuous dosing of ˙OH radicals in seawater as a sustainable approach for AGD treatment. In real life, there will be a considerable load of microorganisms present in both seawater and freshwater, whose cell defense mechanisms against a radical attack would likely be different from a whole animal model. Hence, it is anticipated that continuous dosing of ˙OH radicals would be more detrimental to wild amoebae species including NP in salmon gills in aqueous media than to a complete salmon.

Data Availability

The .xlsx data extracted from BD Accuri C6 software used to support the findings of this study are included within the supplementary information file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

Supplementary Materials

The supplementary section contains information of approximate concentration of clonal NP (Figure S1); wild amoebae species subject to hydroxyl radical bathing in seawater (Figures S2-S3); qPCR cycle threshold data after treating wild amoebae species in different hydroxyl concentrations (Figure S4); qPCR cycle threshold data after treating clonal NP in 35 mM hydroxyl concentration (Figure S5); viability count via flow cytometry after treating clonal NP in 35 mM hydroxyl concentration in seawater for 1 hour (Figure S6); viability count via flow cytometry after treating CHSE-214 cell lines in 35 mM hydroxyl concentration in seawater for 1 hour (Figure S7); toxicity results of 35 mM ˙OH radicals and H_2O_2 treatment towards CHSE-214 in freshwater and seawater at 18°C (Figure S8) and at 15°C (Figure S9); gill score information (Table S1); and 18S primer sequence used for quantitative PCR analyses of wild amoebae collected from salmon gills (Table S2). (*Supplementary Materials*)

References

[1] A. P. Shinn, J. Pratoomyot, J. E. Bron, G. Paladini, E. E. Brooker, and A. J. Brooker, "Economic costs of protistan and metazoan parasites to global mariculture," *Parasitology*, vol. 142, no. 1, pp. 196–270, 2015.

[2] C. J. English, F. Swords, J. K. Downes et al., "Prevalence of six amoeba species colonising the gills of farmed Atlantic salmon with amoebic gill disease (AGD) using qPCR," *Aquaculture Environment Interactions*, vol. 11, pp. 405–415, 2019.

[3] M. D. Powell, P. Reynolds, and T. Kristensen, "Freshwater treatment of amoebic gill disease and sea-lice in seawater salmon production: considerations of water chemistry and fish welfare in Norway," *Aquaculture*, vol. 448, pp. 18–28, 2015.

[4] P. Mahbub and A. Sharma, "Investigation of alternative water sources for fish farming using life cycle costing approach: a case study in North West Tasmania," *Journal of Hydrology*, vol. 579, Article ID 124215, 2019.

[5] J. W. Wynne, C. Stratford, J. Slinger et al., "The interaction between temperature and dose on the efficacy and biochemical response of Atlantic salmon to hydrogen peroxide treatment for amoebic gill disease," *Journal of Fish Diseases*, vol. 43, no. 1, pp. 39–48, 2020.

[6] C. C. Lazado, G. Timmerhaus, L.-F. Pedersen et al., "Peracetic acid as a potential treatment for amoebic gill disease (AGD) in Atlantic salmon - Stage," *Nofima rapportserie*, vol. 1, no. 21, 2019.

[7] R. S. Taylor, J. Slinger, P. Camargo Lima et al., "Evaluation of sodium percarbonate as a bath treatment for amoebic gill disease in Atlantic salmon," *Aquaculture Research*, vol. 52, no. 1, pp. 117–129, 2020.

[8] M. B. Adams, P. B. B. Crosbie, and B. F. Nowak, "Preliminary success using hydrogen peroxide to treat Atlantic salmon, Salmo salar L., affected with experimentally induced amoebic gill disease (AGD)," *Journal of Fish Diseases*, vol. 35, no. 11, pp. 839–848, 2012.

[9] P. Mahbub, A. Smallridge, A. Irtassam, and T. Yeager, "Scalable production of hydroxyl radicals (˙OH) via homogeneous photolysis of hydrogen peroxide using a continuous-flow photoreactor," *Chemical Engineering Journal*, vol. 427, Article ID 131762, 2022.

[10] J. Hudson, M. Adams, K. Jantawongsri, T. Dempster, and B. F. Nowak, "Evaluation of low temperature and salinity as a treatment of atlantic salmon against amoebic gill disease," *Microorganisms*, vol. 10, no. 2, p. 202, 2022.

[11] S. D. Foster, D. A. Griffin, and P. K. Dunstan, "Twenty years of high-resolution sea surface temperature imagery around Australia: inter-annual and annual variability," *PLoS One*, vol. 9, no. 7, Article ID e100762, 2014.

[12] C. Collins, M. Hall, D. Bruno et al., "Generation of Paramoeba perurans clonal cultures using flow cytometry and confirmation of virulence," *Journal of Fish Diseases*, vol. 40, no. 3, pp. 351–365, 2017.

[13] R. N. Borazjani, L. L. May, J. A. Noble, S. V. Avery, and D. G. Ahearn, "Flow cytometry for determination of the efficacy of contact lens disinfecting solutions against Acanthamoeba spp," *Applied and Environmental Microbiology*, vol. 66, no. 3, pp. 1057–1061, 2000.

[14] F. Mi-ichi, Y. Miyake, V. K. Tam, and H. Yoshida, "A Flow Cytometry method for dissecting the cell differentiation process of entamoeba encystation," *Frontiers in Cellular and Infection Microbiology*, vol. 8, p. 250, 2018.

[15] A. R. Bridle, D. L. Davenport, P. B. B. Crosbie, M. Polinski, and B. F. Nowak, "Neoparamoeba perurans loses virulence during clonal culture," *International Journal for Parasitology*, vol. 45, no. 9-10, pp. 575–578, 2015.

[16] R. S. Taylor, J. Slinger, C. Stratford, M. Rigby, and J. W. Wynne, "Evaluation of the infectious potential of Neoparamoeba perurans following freshwater bathing treatments," *Microorganisms*, vol. 9, no. 5, p. 967, 2021.

[17] C. J. English, T. Tyml, N. A. Botwright et al., "A diversity of amoebae colonise the gills of farmed Atlantic salmon (Salmo salar) with amoebic gill disease (AGD)," *European Journal of Protistology*, vol. 67, pp. 27–45, 2019.

[18] D. G. Capone and R. P. Kiene, "Comparison of microbial dynamics in marine and freshwater sediments: contrasts in anaerobic carbon catabolism," *Limnology & Oceanography*, vol. 33, no. 4, pp. 725–749, 1988.

[19] F. Belloc, P. Dumain, M. R. Boisseau et al., "A flow cytometric method using Hoechst 33342 and Propidium Iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells," *Cytometry*, vol. 17, no. 1, pp. 59–65, 1994.

[20] B. F. Nowak, P. Crosbie, and M. Adams, *FRDC Salmon Subprogram - Hydrogen Peroxide Treatment of Atlantic salmon Affected by AGD (2010/218*, University of Tasmania, Hobart, Australia, 2011.

[21] A. T. Wood, R. S. Taylor, P. R. Quezada-Rodriguez, and J. W. Wynne, "Hydrogen peroxide treatment of Atlantic salmon temporarily decreases oxygen consumption but has negligible effects on hypoxia tolerance and aerobic performance," *Aquaculture*, vol. 540, Article ID 736676, 2021.

[22] P. Pędziwiatr, F. Mikołajczyk, D. Zawadzki, K. Mikolajczyk, and A. Bedka, "Decomposition of hydrogen peroxide - kinetics and review of chosen catalysts," *Acta Innovations*, vol. 26, pp. 45–52, 2018.

[23] C. Marzzacco, "The effect of a change in the catalyst on the enthalpy of decomposition of hydrogen peroxide," *Chemistry*, vol. 12, no. 360, pp. 12-13, 2008.

[24] D. C. Harris, *Quantitative Chemical Analysis*, W. H. Freeman and Company, New York, NY, USA, 7th edition, 2007.

[25] I. Cano, N. G. H. Taylor, A. Bayley et al., "In vitro gill cell monolayer successfully reproduces in vivo Atlantic salmon host responses to Neoparamoeba perurans infection," *Fish & Shellfish Immunology*, vol. 86, pp. 287–300, 2019.

[26] A. Butler, "Acquisition and utilization of transition metal ions by marine organisms," *Science*, vol. 281, no. 5374, pp. 207–209, 1998.

[27] K. V. Brix, D. K. DeForest, M. Burger, and W. J. Adams, "Assessing the relative sensitivity of aquatic organisms to divalent metals and their representation in toxicity datasets compared to natural aquatic communities," *Human and Ecological Risk Assessment: An International Journal*, vol. 11, no. 6, pp. 1139–1156, 2005.