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

Electronic Supplementary Information

Prospect of hydroxyl radical exposure during seawater bathing to treat Amoebic Gill Disease in Atlantic salmon

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S1. Wild *Neoparamoeba Perurans* (NP) collection and transfer from gills

The gill basket of at least six farmed Tasmanian Atlantic salmon that displayed clinical signs of AGD with a gill score greater than 3 were dissected. Individual gills were isolated and collected in 50 mL falcon tubes and transported from Huon's Atlantic salmon aquaculture facilities in Tasmania to VU laboratory in Melbourne in seawater within the same day of collection at 2-4 °C. The seawater was collected from the pens where salmon is farmed. The pH and salinity of the seawater during collection were recorded. The gill scores were observed according to the following Table (adapted from Taylor et al. 2009):

Table S1 Gill Scores of AGD infected salmons according to Taylor et al. (2009)

Infection level	Gill score	Gross description
Clear	0	No sign of infection and healthy red colour
Very light	1	1 white spot, light scarring or undefined necrotic streaking
Light	2	2–3 spots/small mucus patch
Moderate	3	Established thickened mucus patch or spot groupings up to 20% of gill area
Advanced	4	Established lesions covering up to 50% of gill area
Heavy	5	Extensive lesions covering most of the gill surface

We followed the protocol below for collecting the wild NP from salmon gills:

1. Upon receipt of the gill samples, we transferred the gills into T75 culture flasks in 25 mL seawater and placed on automatic shaker for 60 minute shake.
2. We scraped the gill mucus with a sterile scraper, collected the mucus in the T75 flask and discarded the gill.
3. We checked the seawater in the T75 flask to confirm floating and settled NP species in the T75 flask.
4. We allowed 15-20 minutes for NP to settle at the bottom before discarding the floating mucus and other gill debris. Then we added 18 mL fresh 35 ppt seawater.

5. We also centrifuged the seawater in 50 mL falcon tubes that contained the gills at 3000 x g for 5 minutes and discarded the supernatant. We re-suspended the pellets in 2 mL 35 ppt seawater and added it to the already existing 18 mL seawater in the T75 flask prepared in step 4.
6. Finally, we added 200 µL 10X MYB to the T75 flask and incubated the flask in a wine cooler at 14° C and media changed the flask every five days. We passaged the NP in every three week by centrifuging at 3000 x g for 5 min at suitable dilution.
7. We used any T75 flask for our experiments when the NP confluence reached 80% in that flask. We applied 10X pen/strep two days prior to any experiment.

S1.1. Protocols for clonal NP transfer on CHSE-214 cell lines

We received clonal NP culture from CSIRO after Victoria University (VU) signed a material transfer agreement with CSIRO. Figure S1 shows the 50 mL Falcon tubes filled with clonal NP sent to VU.

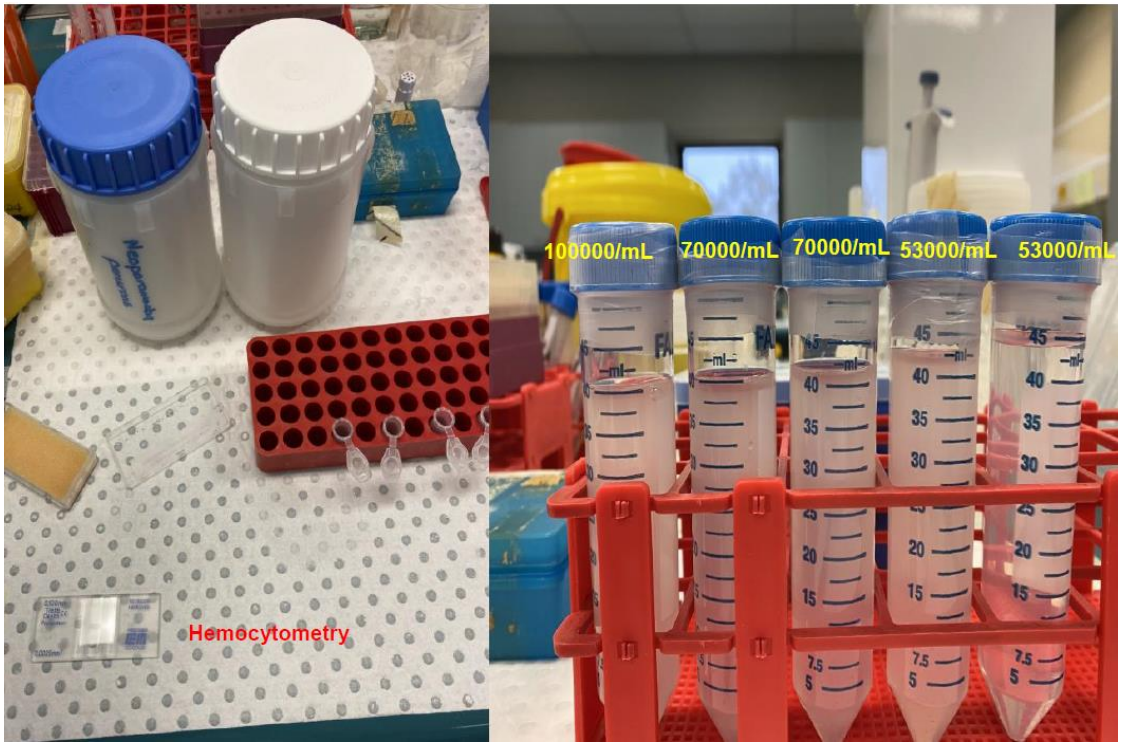

Figure S1 clonal NP species supplied in 36 ppt seawater in 50 mL falcon tubes. Approximate concentrations were determined through hemocytometry.

After gently shaking the falcon tubes, we transferred the NP samples from flacon tubes into two T75 culture flasks by equally pouring 25 mL into each flask. We incubated the T75 flasks in a wine cooler at 14° C. We added 200 µL 10X MYB in each T75 flask and media changed the flask every five days. When the NP confluence reached 80% in a flask we applied 10X pen/strep two days prior to transferring the NP on another T75 flask containing 80% confluent CHSE-214 cell lines. Before transferring the NP, we gently shook the T75 flasks with NP side to side for 5 minutes. We carefully discarded the media of the CHSE-214 and then poured the NP in seawater into the CHSE-214 flask. The NP settled on the CHSE-214 cell lines within 5 minutes after transfer which was confirmed under microscope. We immediately started our hydroxyl (·OH) radical and hydrogen peroxide (H_2O_2) exposure experiments on these T75 flasks after NP settles on CHSE-214 cell lines

S1.2. Protocols for Continuous dosing of ·OH radicals and H_2O_2 at different temperatures

We have undertaken our experiments in the following manner:

1. Culture Chinook Salmon embryo cell line (CHSE-214) in T-75 culture flasks (maximum capacity 75 mL) according to the vendor specified culture protocols as adherent cells (http://www.cellbankaustralia.com/chse-214.html).
2. Set the incubator temperature at 18 °C and 15 °C to conduct separate sets of experiments at these pre-defined temperatures.
3. Replace the culture media with either 5mL fresh seawater or 5 mL riverwater and add 11.9 mL 50 mM hydroxyl or 11.9 mL 50 mM hydrogen peroxide to bring down the concentration to 35 mM at the start of the bathing. Collect 1.5 mL samples from each flask at the start of bathing for measuring residual hydrogen peroxide in each flask.
4. Continuously dose the culture flasks at 200 microlitre/min with either 35 mM OH or 35 mM H2O2 and collect 1.5 mL samples at 1 hr, 1.5 hr, 2 hr and 4 hr in order to check the hydrogen peroxide and hydroxyl radical levels.
5. Take microscope images of the culture flasks at the beginning of bathing, at 1 hr, 1.5 hr, 2 hr and 4 hr durations.
6. At the end of 4 hr bathing session, collect the media in 50 mL falcon tubes
7. Add 2 mL 0.25% trypsin to each of the T75 culture flasks and shake for 5 minutes followed by addition of 5 mL minimum essential medium (GIBCO) enriched with 10X foetal bovine serum and 50X penicillin/streptomycin and 10 X non-essential amino acids.
8. Collect the solution in 10 mL centrifuge tubes and pellet cells at 500 x g for 5 min; discard the supernatant and resuspend the pellets in 2 mL of either riverwater or seawater.

9. Collect 1 mL of the solution for subsequent flow cytometry to measure percent viability of the salmon cells after 4 hr bathing. Target cell concentration for flow cytometry was 100000 – 300000 cells/mL in the T75 flasks which was confirmed by hemocytometry. We measured the percent viability of these cells in the worst case scenario of 4 hr bathing.
10. Each experiment was accompanied with a control flask which was not subjected to any treatment at all, but the media is replaced with equal volume of either seawater or riverwater at the start of the treatment at corresponding temperatures. This flask is referred to as "control flask". The other treatment flasks are referred to as "OH flask" and "H_2O_2 flask". These two flasks contained 35 mM OH radical and H_2O_2, respectively from the beginning of the bathing and were continuously dosed with the 35 mM OH radical or H_2O_2 at a flow rate of 200 microlitre/min.

S2. Toxicity results on wild amoebae adhering to salmon cells via microscopy

We investigated the effects of 35 mM (595 ppm) OH radicals on wild amoebae (supplied by Huon via AGD infected salmon gills) adhering to CHSE-214 salmon embryonic cells and allowed 1-hour bathing in 24-well plates. We counted the cells using hemocytometry and there were ~20,000 wild NP cells/mL. In this case, we used 0.02% malt yeast broth (MYB) in 35 ppt seawater (autoclaved and 0.45 µm filtered) supplied to us by Huon as the growth media for wild NP. We employed 10,000 units penicillin and 10 mg streptomycin/mL (100X pen-strep) diluted to 10X and applied 20 µL of 10X pen-strep to our wild NP samples 2 days prior to test. Figures S2 and S3 demonstrating the wild amoebae cell before and after the 35 mM (595 ppm) OH radical treatment for 60 minute.

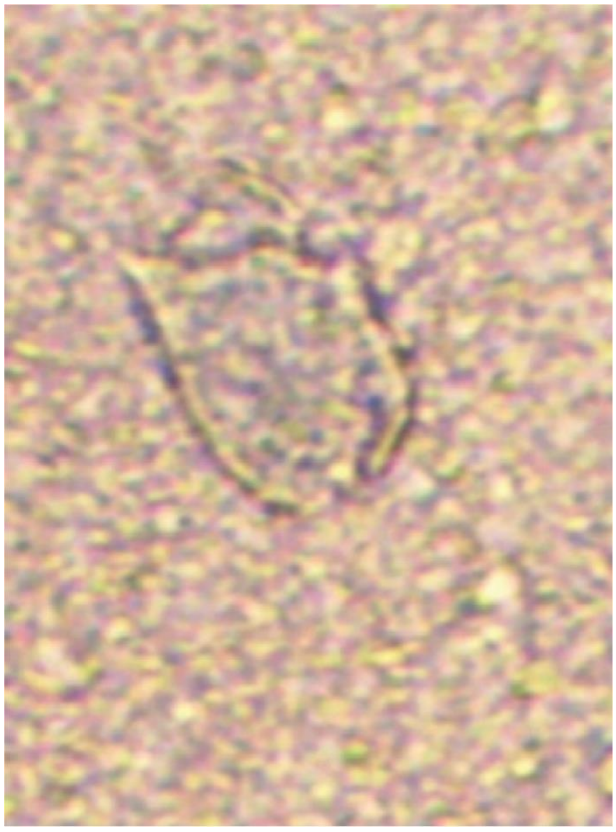

Figure S2 A healthy single wild amoeba sitting on top of CHSE-214 embryonic cells 24 hours after the media for CHSE-214 has been replaced with 0.02% MYB media in 35 ppt seawater. Magnification: 400X.

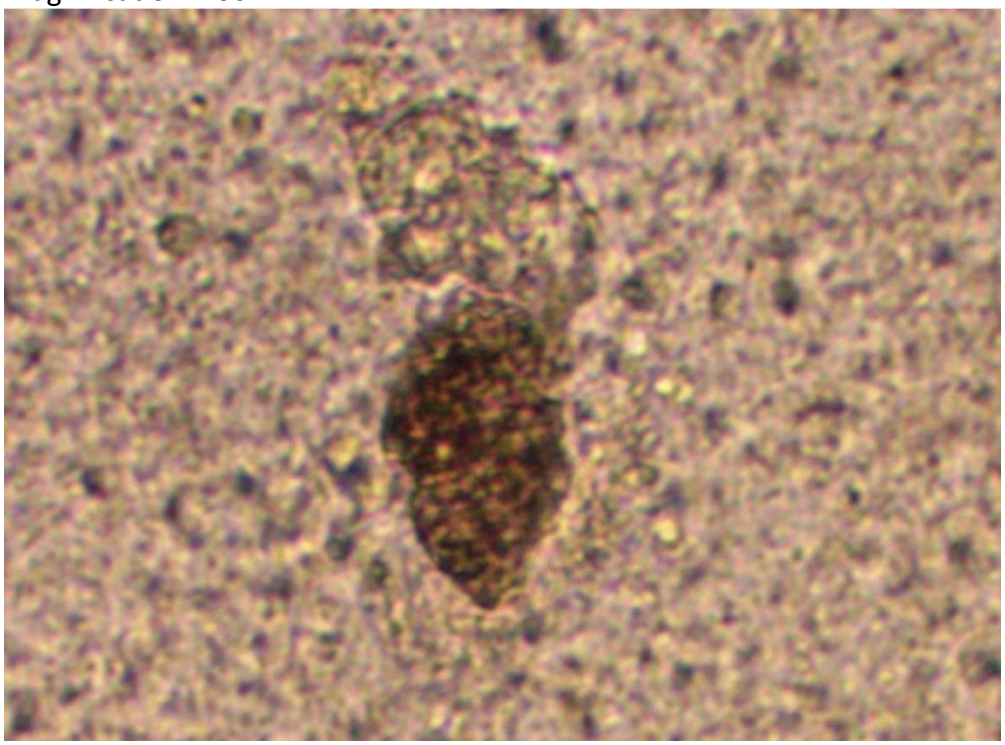

Figure S3 A distressed single wild amoeba sitting on top of CHSE-214 embryonic cells exposed to 35 mM OH radical and left for bathing in 35 ppt seawater for 60 minutes in 35 mM OH radical in the seawater media. Magnification: 400X.

In Figure S3, we observe that the wild amoeba cell is not looking healthy with evidence of damages of the cell boundary after the 60-minute bathing in 35 mM (595 ppm) OH radicals in 0.02% MYB in filtered seawater media.

S3. Protocols for quantitative PCR (qPCR) and Gel Electrophoresis (GE)

we utilized a universal primer (based on 18S ribosomal RNA , please refer to Table 2) for eukaryotes especially amoeba in the sample on the basis of previous studies undertaken for sequencing of marine amoebae by Smirnov et al. (2007), as well as for sequencing of amoebae colonizing AGD infected salmon gills by English et al. (2019). DNA was extracted from the samples using the Wizard Genomic DNA Purification kit (Promega) following the recommended protocol. To date there is no Chinook salmon 18S gene sequence published. According to Murata et al. (1993) the closest phylogenetic match is Coho salmon (XR_004209003). This sequence was analysed for unique primer pairs using standard parameters at NCBI Primer-BLAST. Primers were synthesized by Integrated DNA Technologies Australia Pty Ltd for use in qPCR (Table S2). Each reaction was carried out with 4 µL template per reaction, 1 µL 1X forward primer, 1 µL 1X reverse primer, 12.5 µL SsoAdvanced Universal SYBER Green Supermix (BioRad) and made up to 25 µL with sterile deionized water. The samples were subject to qPCR on a BioRad CFX96 qPCR machine with the following parameters: amplification 95°C for 3 min, denaturation at 95°C for 30 sec, annealing at 50°C for 2 min, and extension at 72°C for 2 min, repeat 34x, with a final extension at 72°C for 5 min, then held at 12°C. Lid temperature was 105°C. All qPCR data was confirmed by traditional gel electrophoresis on a 1% agarose gel in 1x TAE buffer with 8 µL SYBR safe DNA gel stain (Thermo Fisher) We loaded 5 µL samples and 100 bp DNA molecular ruler (BioRad #1708202) into the wells and applied 70 Volts for 60 minutes during gel electrophoresis.

Table S2 18S primer sequence used for quantitative PCR analyses of wild amoebae collected from salmon gills and CHSE-214 cell lines

Sequence	**Name**	**Species**	**Scale**	**Product size**	**Reference**
TGATCCATCTGCAGGTTCACCTAC	RibB Forward	NP	25 nmole	800 bp	English et al. (2019)[14]
GATYAGATACCGTCGTAGTC	S12.2 Reverse	NP	25 nmole		
AACGGCTACCACATCCAAGG	Forward PP2	CHSE-214	25 nmole	245 bp	XR_004209003
CCCGAGATCCAACTACGAGC	Reverse PP2	CHSE-214	25 nmole		

S3.1. In vitro toxicity of hydroxyl radicals to wild amoebae species collected from salmon gills as well as to clonal NP collected from CSIRO

Wild amoebae species originating from AGD affected Atlantic salmon gills were exposed to 10 mM (170 ppm), 25 mM (425 ppm), 35 mM (595 ppm) and 50 mM (850 ppm) ·OH radicals for 1 hr. The qPCR results of wild amoebae and clonal NP are shown in Figure S4 and Figure S5, respectively.

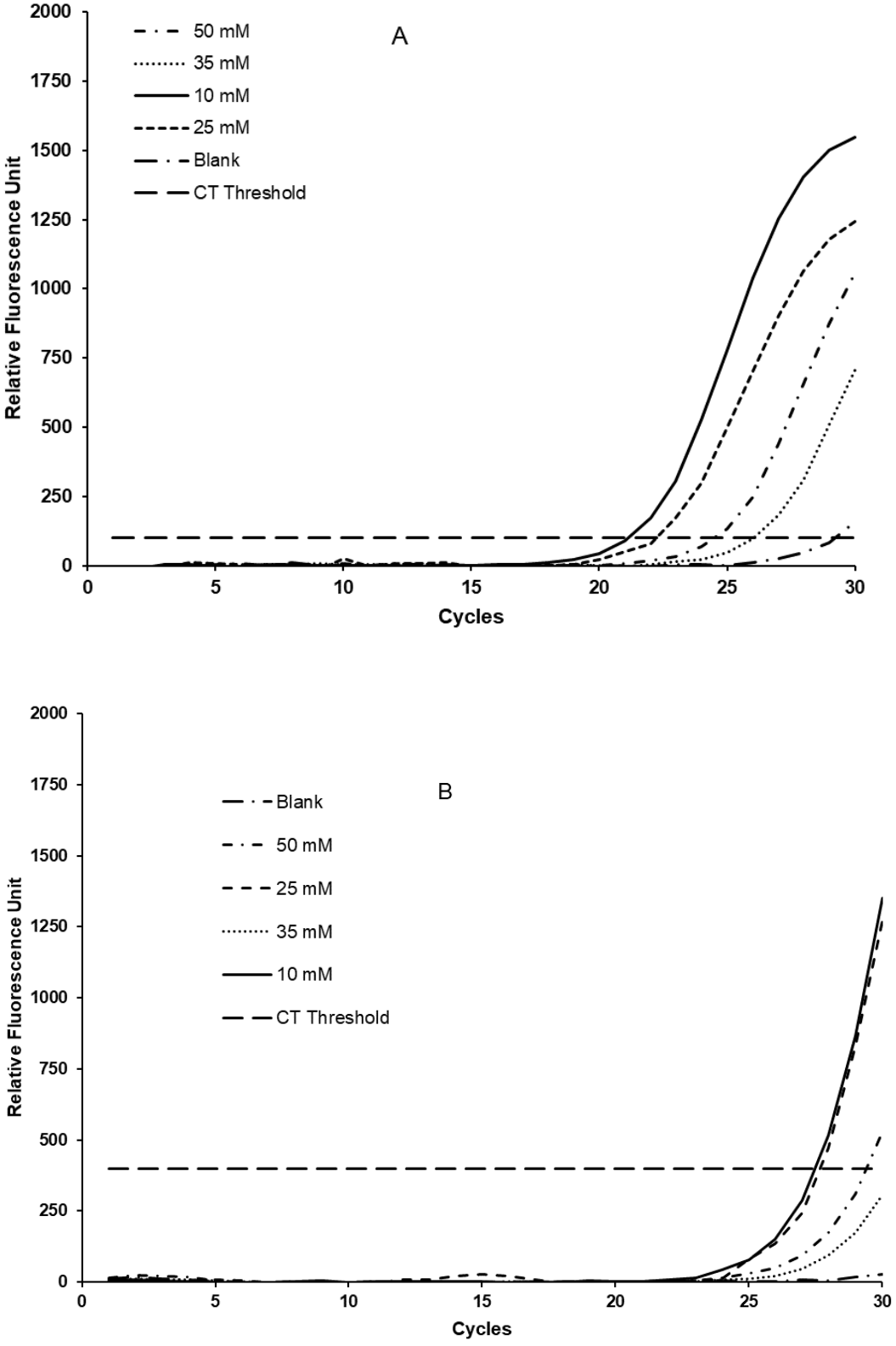

Figure S4: Recovery of wild *amoebae collected from AGD affected salmon gills* treated with hydroxyl radicals from 10 mM to 50 mM as determined by comparative cycle threshold (ct) values obtained by quantitative PCR (qPCR). (A) Observation 1 at the end of 1 hr treatment and (B) observation 2 at the end of 7 days after treatment.

In Fig. S4 (A), we observe that increased ·OH radical concentrations from 10 mM (170 ppm) up to 35 mM (595 ppm) resulted in the corresponding ct value moving towards the negative qPCR control ct value, indicating increased damage to DNA in the sample. We accept the fact that there could be some contaminations in our qPCR negative control that was made of 0.25µm filtered and autoclaved seawater. Xiao et al. (2019) have highlighted that microbial physiological dysfunction by ·OH radicals takes place via three main pathways: damage to the cell membrane/wall, enzymes and genetic material such as DNA and RNA. Several reports also identify ·OH radical generated inactivation sites in bacteria and virus (Cho et al. 2005), as well as algae (Wu et al. 2011). However, no reports to date have elucidated the possible genetic damage to amoebae species caused by ·OH radicals. Our data indicates that wild amoebae are not recovering in the 7 days post-treatment (Fig S4 (B)) as indicated by the increased ct values compared to observation 1 after 1 hr ·OH radical treatment. This suggests gradual damage to DNA resulting from applied ·OH radical concentrations in this controlled experiment.

We also completed qPCR analysis of clonal NP before and after ·OH radical treatment (Fig. S5). We observed the qPCR ct values increases following treatment with 35 mM ·OH radicals compared to the no treatment control, indicating damage to DNA post treatment (Fig S5).

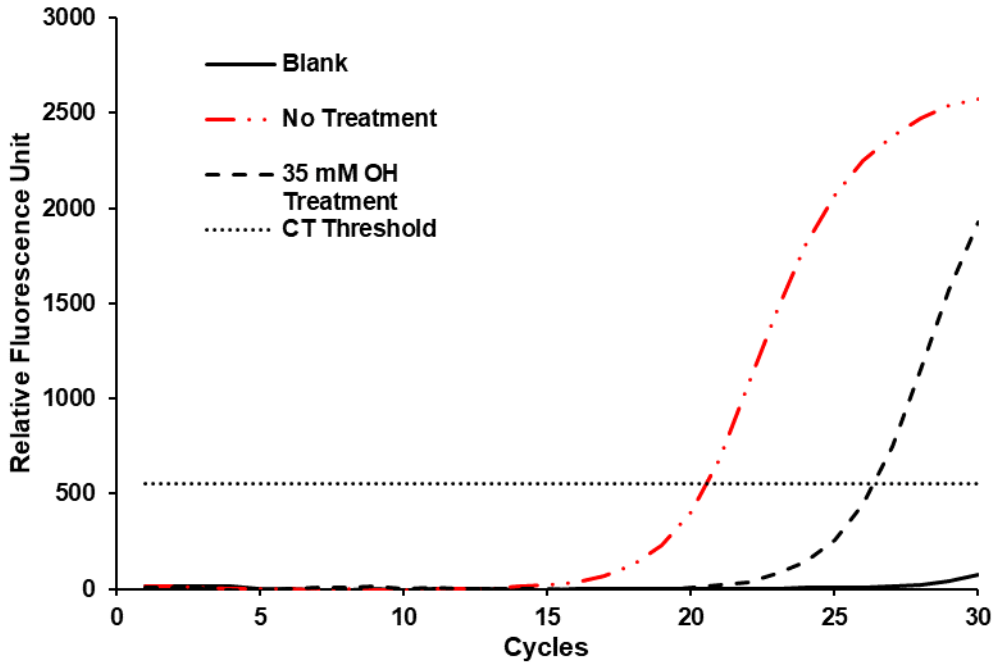

Figure S5: Treatment of clonal NP cells adhering to CHSE-214 cells treated with 35 mM (595 ppm) hydroxyl radicals for 60 minutes. Although there could be some contaminations in the qPCR negative control that was made of 0.25µm filtered and autoclaved seawater, the qPCR ct values increased with treatment, indicating damage to the DNA materials of clonal NP.

S4. Repeated experiments on viabilities of clonal NP and CHSE-214 assessed by Flow Cytometry

We repeated the experiments described in sections 3.3 and 3.4 in the main manuscript for 1 hr treatment at 35 mM ·OH radicals only in seawater at 15 °C for both clonal NP and CHSE-214 cell lines. Figure S6 shows the flow cytometry results of clonal NP.

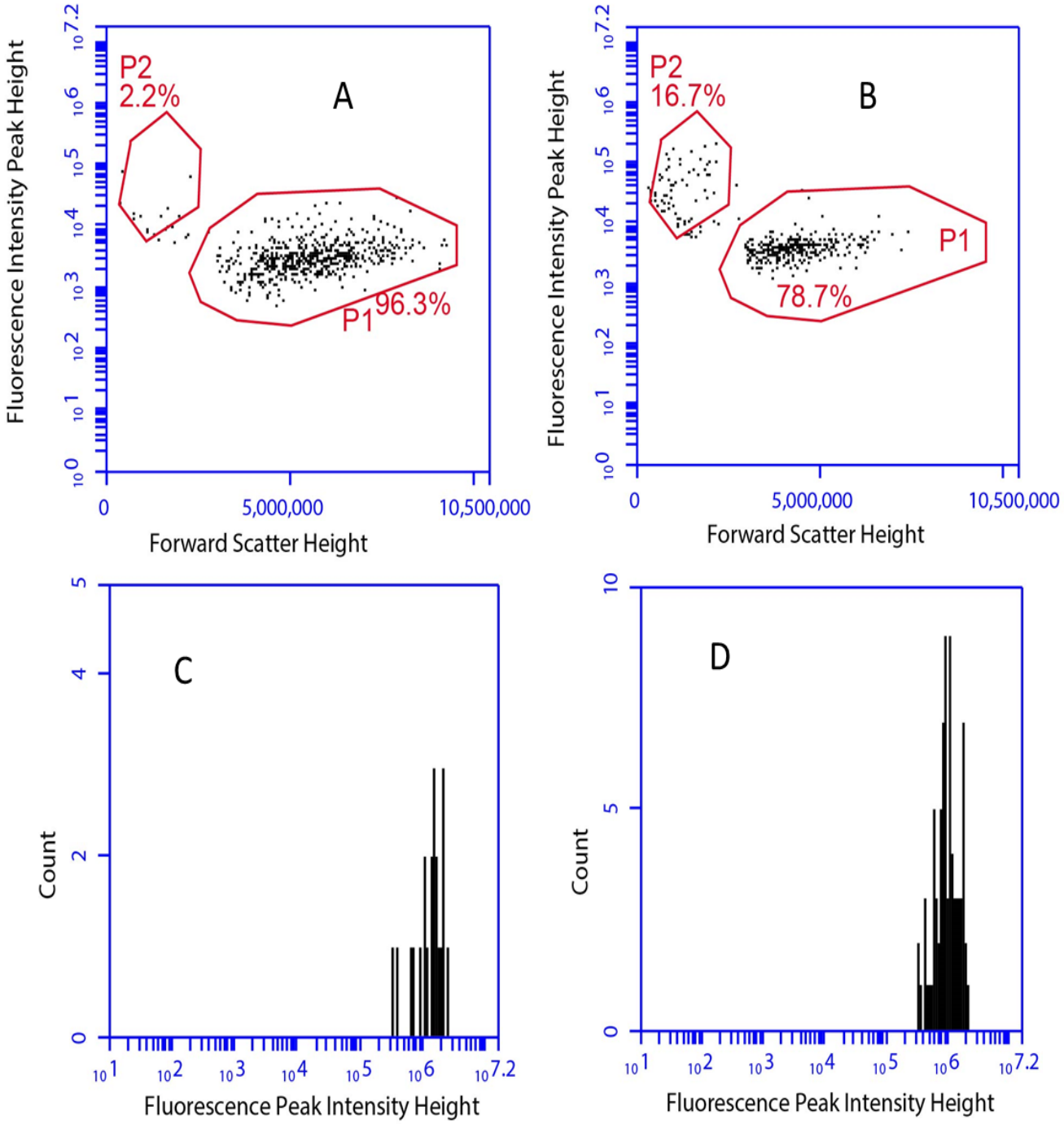

Figure S6 (A) Live clonal NP cells (polygon P1) and dead clonal NP cells (polygon P2) before 35 mM OH radical treatment for 1 hr in seawater; (B) Live clonal NP cells (polygon P1) and dead clonal NP cells (polygon P2) after 35 mM OH radical treatment for 1 hr in seawater showing 16.7% dead NP after treatment; (C) Histogram of dead NP (total dead count = 13 out of 597 NP cells) before treatment; (D) Histogram of dead NP (total dead count = 65 out of 389 NP cells) after treatment. Thus, the cytometry shows approximately 17% of the total NP population was dead after the treatment which is close to ~22.5% found dead in Figure 4 in main paper.

Figure S7 shows the flow cytometry results of viabilities of CHSE-214 cell lines after exposed to 1 hr treatment in seawater in 35 mM ·OH radicals at 15 °C.

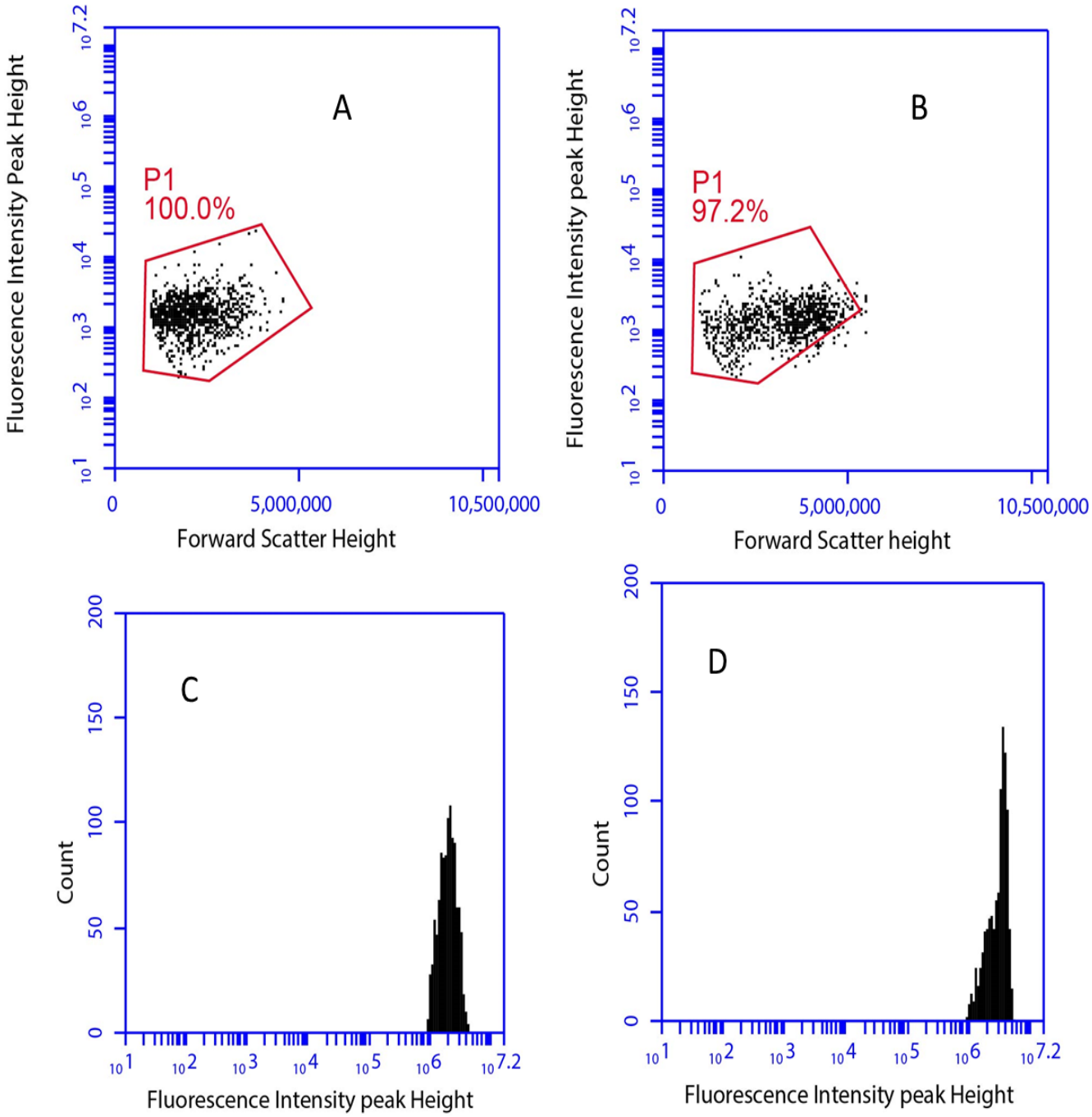

Figure S7 (A) Live CHSE-214 cell lines (polygon P1) before 35 mM OH radical treatment for 1 hr in seawater; (B) Live CHSE-214 cell lines (polygon P1) after 35 mM OH radical treatment for 1 hr in seawater after treatment; (C) Histogram of live CHSE-214 (total live count = 1091) before treatment; (D) Histogram of live CHSE-214 (total live count = 988) after treatment.

Thus, the survival rate of CHSE-214 after 1 hr treatment with OH radical in seawater accounts for approximately 90%. This finding is expected as we saw in Figure 5 in main paper that irrespective of media and temperature the survival rate of CHSE-214 reduced with increased treatment time of more than 1 hour (about 60% for 4 hr treatment).

S5. Flow Cytometry Results of 35 mM OH and H2O2 treatment towards CHSE-214 in Fresh and Seawater

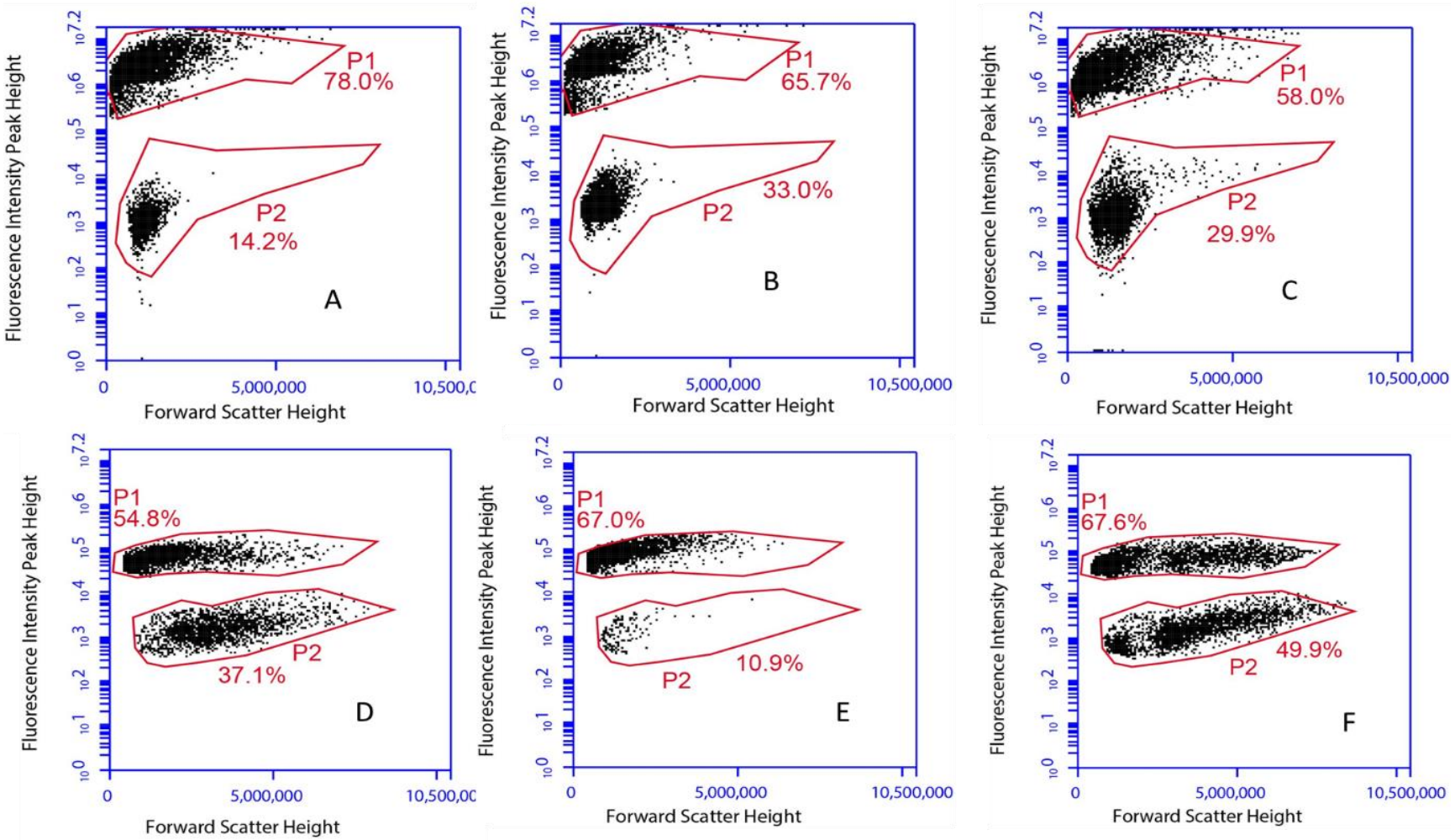

Figure S8 (A) FW 18 °C experiment of Fig 5; P1=live % of CHSE 214 after .OH treatment, P2= dead % of CHSE 214 after .OH treatment, (B) P1=live % of CHSE 214 after H2O2 treatment, P2= dead % of CHSE 214 after H2O2 treatment, and (C) P1=live % of CHSE 214 in control flask

after treatment duration of 1.5 hr, P2= dead % of CHSE 214 in control flask after treatment duration of 1.5 hr (Control flasks were not subject to any treatment); (D) SW 18 °C experiment of Fig 5; P1=live % of CHSE 214 after .OH treatment, P2= dead % of CHSE 214 after .OH treatment; (E) P1=live % of CHSE 214 after H2O2 treatment, P2= dead % of CHSE 214 after H2O2 treatment; (F) P1=live % of CHSE 214 in control flask after treatment duration of 2 hr, P2= dead % of CHSE 214 in control flask after treatment duration of 2 hr (Control flasks were not subject to any treatment).

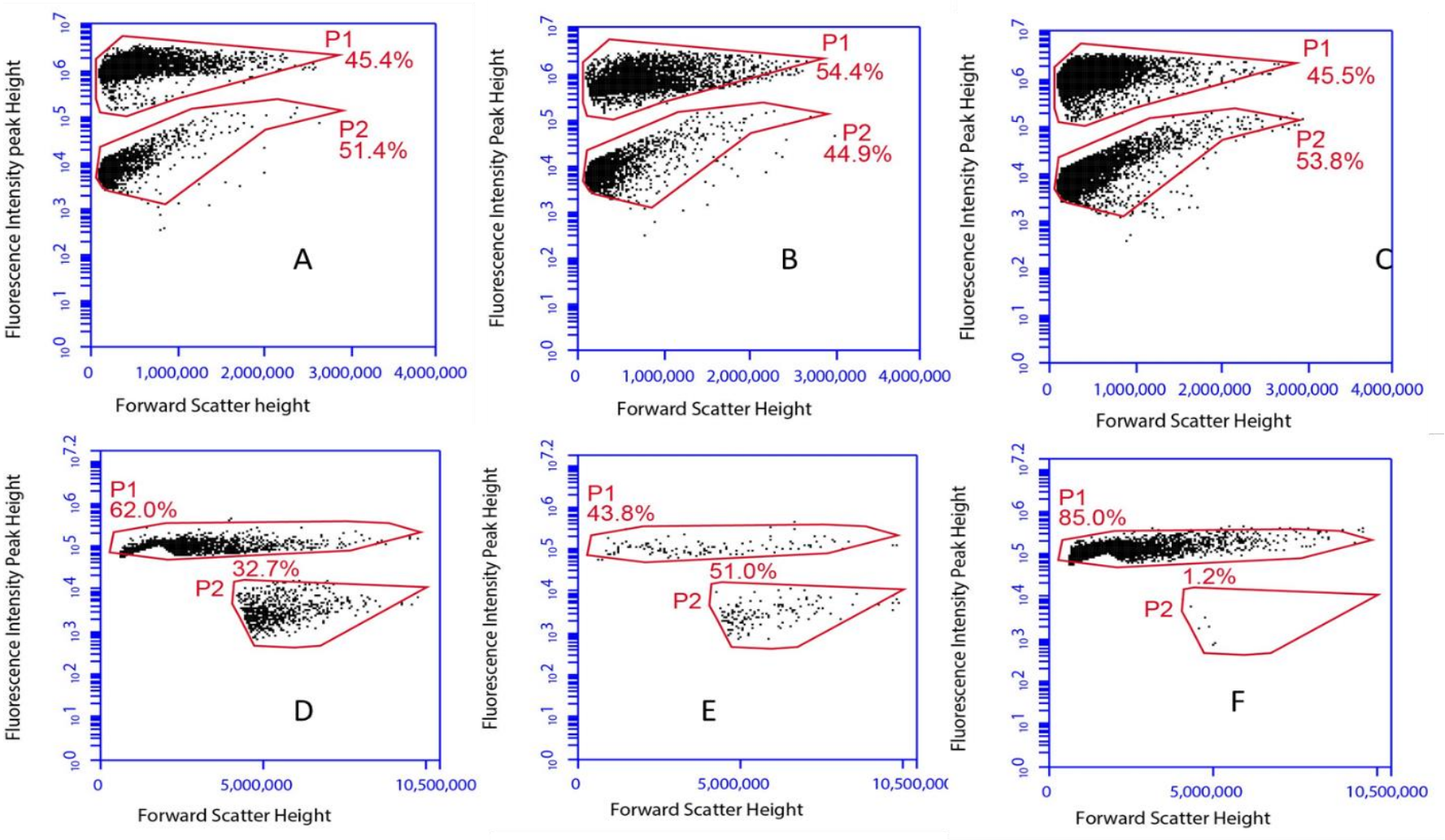

Figure S9 (A) FW 15 °C experiment of Fig 5; P1=live % of CHSE 214 after .OH treatment, P2= dead % of CHSE 214 after .OH treatment, (B) P1=live % of CHSE 214 after H2O2 treatment, P2= dead % of CHSE 214 after H2O2 treatment, and (C) P1=live % of CHSE 214 in control flask

after treatment duration of 2 hr, P2= dead % of CHSE 214 in control flask after treatment duration of 2 hr (Control flasks were not subject to any treatment); (D) SW 15 °C experiment of Fig 5; P1=live % of CHSE 214 after .OH treatment, P2= dead % of CHSE 214 after .OH treatment; (E) P1=live % of CHSE 214 after H2O2 treatment, P2= dead % of CHSE 214 after H2O2 treatment; (F) P1=live % of CHSE 214 in control flask after treatment duration of 4 hr, P2= dead % of CHSE 214 in control flask after treatment duration of 4 hr (Control flasks were not subject to any treatment).

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