Response of *Deinococcus radiodurans* and desert crust isolate *Patulibacter americanus* to H_2O_2 exposure

Honors Thesis

Olivia Faye Scott

Thesis Advisor: Dr. Laurie Caslake

Thesis Committee: Dr. Laurie Caslake, Dr. Daniel Strömbom, and Dr. Michael Butler

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ABSTRACT

Biological soil crusts (BSCs) are layers of microbial and multicellular life that perform crucial ecosystem functions in arid and semi-arid environments. Increased desertification and human disturbance of BSCs warrant study into their ability to withstand and recover from desiccation-induced oxidative stress by their environment. Previous research on oxidative stress has found that reactive oxygen species (ROS) cause extensive damage to cell biomolecules, including DNA. *Deinococcus radiodurans,* an extremely oxidative stress-tolerant bacterium, has many DNA repair mechanisms that have been well-studied, but there are very few studies analyzing the repair mechanisms in other organisms, with the exception of *Escherichia coli*. This study aims to compare *Patulibacter americanus,* a BSC isolate, to *D. radiodurans* to determine differences in survival and recovery from oxidative stress induced by ROS hydrogen peroxide (H_2O_2). It was found that *P. americanus* exhibits greater survival after H_2O_2 treatment than *D. radiodurans,* and that growth after removal of treatment was largely unaffected. Additionally, it appears that *D. radiodurans,* but not *P. americanus,* sustained damage to DNA as a consequence of H_2O_2 treatment. Overall, this investigation provides evidence that *P. americanus* exhibits greater oxidative stress tolerance than *D. radiodurans,* suggesting that BSC isolates may have specialized adaptations for survival in highly desiccated environments.

INTRODUCTION

Biological Soil Crusts

Biological soil crusts (BSCs) are superficial layers of microbial and multicellular life that are closely associated with soil particles and are common in arid environments (Belnap and Lange, 2003). BSCs consist of cyanobacteria, lichen, fungi, and a variety of other organisms (Moreira-Grez et al., 2019). Nearly 47% of the Earth's land surface is arid (Soussi et al., 2016); BSCs cover up to 70% of areas of sparse vegetation in these regions and perform crucial ecosystem functions, including carbon and nitrogen fixation, mineralization, and soil moisture retention (Ghiloufi et al., 2019; Castillo-Monroy and Maestre, 2011).

In the arid environments where BSCs can be found, there are few, if any, vascular plants. In most ecosystems, vascular plants make up a large portion of the primary producer segment of the food pyramid, providing the essential organic carbon that higher-order organisms are not capable of synthesizing on their own. However, in arid environments, the ability of vascular plants to fix carbon by performing photosynthesis is limited by the low water content. BSCs crucially fix carbon into the surrounding soil, allowing for vascular plant life to take root and form the base of the food pyramid; in fact, vascular plants growing within BSCs tend to have a higher biomass and greater nutrient content than those grown in uncrusted soils (Belnap, 2003). BSCs in the United States have become a focus of recent study; depending on a variety of factors, such as potential evapotranspiration, soil characteristics, and climate, it is estimated that their recovery from increasing human interference and the damaging effects of climate change could take anywhere between 20 and 1000 years (Belnap, 2003). Given the difficulties of recovery, their importance to their ecosystems, and the rising threats they face in an

anthropogenic world, BSCs are an essential area of study in ecology, microbiology, conservation biology, and a variety of other fields.

Desiccation

Since many BSCs are situated within arid environments, they are frequently subjected to desiccation (Belnap, 2003). BSCs are highly resistant to long periods of drought and can survive dry weight water contents as low as 5% by halting all metabolic processes (Bewley and Krochko, 1982). BSCs withstand and recover from long periods of drought by creating extracellular polysaccharide matrices and increasing concentrations of compatible solutes, among other strategies (Robertson and Firestone, 1992; Lebre et al., 2017). In spite of this, factors involved in the increased desertification of arid and semi-arid ecosystems threaten to overwhelm BSCs' resilience. The United Nations Convention to Combat Desertification (UNCCD) defines desertification as "land degradation in arid, semi-arid and dry sub-humid areas resulting from various factors, including climatic variations and human activities" (United Nations, 1994). The UNCCD has also estimated that about 25% of Earth's land area is affected by desertification (United Nations, 1994), and research has suggested that this figure will increase in the coming years as the effects of climate change continue to degrade these ecosystems (Reynolds et al., 2007). In addition to habitat loss from physical human disruption (Huang et al., 2020), desertification is one of the major threats to BSCs. Understanding the full mechanism of BSC desiccation resistance is essential in order to predict and prepare for the impending deficits to these important biological structures.

Oxidative Stress

Desiccation, an oxidative stressor, creates a variety of physical, metabolic, biochemical, and physiological stress in bacterial cells (Lebre et al., 2017). Oxidative stress occurs when the balance between ROS production and antioxidant defenses utilized by the cell is disrupted (Ray et al., 2012). Desiccation causes the loss of water molecules that are essential to protein structure and function, leading to both the impairment of important cellular pathways, including those involved with the synthesis, repair, and transport of materials, and the buildup of free radicals (Lebre et al., 2017). This leads to the accumulation of DNA-damaging reactive oxygen species (ROS) within the cell (Lebre et al., 2017).

H_2O_2 is an example of an ROS. Under standard temperature and pressure conditions, H_2O_2 decomposes spontaneously to oxygen and water (Serra-Maia et al., 2018). However, in the presence of iron ions, H_2O_2 is instead converted to either a hydroxyl ion and a hydroxyl radical or a hydrogen ion (a proton) and a hydroperoxyl ion through the Fenton reaction (Figure 1; Wardman and Candeias, 1996; Flowers et al., 1997). A similar reaction can occur with copper (Pham et al., 2013) or other ions of transition metals (Chumakov et al., 2016), also resulting in the formation of radicals. The radicals then react immediately with the nearest molecule at their site of generation, due to their high reactivity (Clark, 2008), resulting in structural changes that affect cell functioning. H_2O_2 is also formed by the dismutation of superoxide, another radical, into molecular oxygen by superoxide dismutase enzymes (Zhao and Drlica, 2014).

ROS can cause several different types of damage to DNA, including oxidation of nitrogenous bases (Girard and Boiteux, 1997), single-strand breaks (Flowers et al., 1997), and double-strand breaks (Karanjawala et al., 2002). Oxidation of nitrogenous bases causes a change in structure that can lead to base change mutations (Neeley and Essigmann, 2006). Single-strand

breaks, the most common type of damage induced by ROS, are caused by the oxidation of the sugar of a nucleotide, leading to the breakage of the phosphodiester bond connecting it to the adjacent nucleic acid (Figure 2; Clark, 2008). Double-strand breaks, although less common than single-strand breaks, are repaired more slowly and are significant problems for cells (Woodbine et al., 2011). Double-strand breaks caused by ROS result in non-ligatable termini due to damage to sugars and bases (Woodbine et al., 2011). Exposure to H_2O_2 is known to cause both single- and double-strand breaks in DNA, at concentrations as low as 15 μM for as little as 30 minutes (Sharma 2016).

Oxidative Stress Tolerance

Pathways and strategies employed by bacterial species for preventing, withstanding, and recovering from oxidative stress have been studied extensively (Fredrickson et al., 2008; Lebre et al., 2017; Archibald and Fridovich, 1982; Slade and Radman, 2011). Overall, bacteria employ lowered metabolism, maximization of water retention and prevention of water loss, and the production of osmoprotectants to prevent damage to DNA and proteins in order to resist oxidative stress caused by desiccation (Lebre et al., 2017). More specific strategies employed include: the cessation of measurable metabolism (García, 2011); accumulation of Mn^{2+}, an effective superoxide scavenger, within the cell (Fredrickson et al., 2008; Archibald and Fridovich, 1982); and the replacement of water molecules with sugars and compatible solutes in biomolecule-stabilizing hydrogen bonds (Potts 1994), which in turn influence the activity of heat shock proteins (Jain and Roy, 2009). The exact mechanisms of many of these strategies are poorly understood (Fredrickson et al., 2008).

H_2O_2 and other ROS are byproducts of mitochondrial oxidative phosphorylation (Ray et al., 2012), the essential last step of cellular respiration involving the transfer of electrons from electron carriers NADH and $FADH_2$ to molecular oxygen via a series of electron-carrying proteins, generating a concentration gradient of protons that powers the production of ATP by ATP synthase (Berg et al., 2002). As a result, aerobic organisms have mechanisms for tolerance or prevention of H_2O_2 formation (Wang and Schellhorn, 1995). The most common and well-studied enzyme involved in this process is catalase, which catalyzes the decomposition of H_2O_2 to water and oxygen (Markillie et al., 1999).

Deinococcus radiodurans

The bacterium *Deinococcus radiodurans* is well-known for its remarkable tolerance to a variety of extreme conditions, including but not limited to UV radiation (Boling and Setlow, 1966), ionizing radiation (Slade and Radman, 2011), oxidizing agents (Wang and Schellhorn, 1995), and desiccation (Mattimore and Battista, 1996). *D. radiodurans,* first isolated from canned meat that had spoiled even after X-rays were used for sterilization (Anderson et al., 1956), is a gram-positive, non-motile, red-pigmented (Minton, 1994), catalase-positive bacterium (Markillie et al., 1999; Jeong et al., 2016). *D. radiodurans* can withstand acute doses of gamma radiation up to 1,500 kilorads (Makarova et al., 2001). *D. radiodurans* is able to begin to repair its genome after extremely high doses of radiation within 3-4 hours, with normal growth resuming afterwards (Cox et al, 2010; Harris et al. 2004). Since this extreme radiation does not occur naturally anywhere on Earth, it has been hypothesized that the species originally developed its extreme tolerance to oxidative stress as an adaptation to desiccation, and that its radiation resistance is secondary to that original function (Mattimore and Battista, 1996). Interestingly, it

has been shown that although *D. radiodurans* exhibits remarkable survival after high doses of radiation, it does not incur less DNA damage than other organisms; therefore, its tolerance of radiation has been attributed to an extremely efficient DNA repair system rather than an ability to avoid DNA damage (Krisko and Radman, 2013).

Many of the desiccation resistance pathways that are identifiable within the genome of *D. radiodurans* are not present in any other bacteria, but *are* present in eukaryotes, suggesting that horizontal gene transfer may have contributed to the organism's remarkable DNA damage repair mechanisms (Makarova et al., 2001). Although it is less common, horizontal gene transfer has been known to occur between bacteria and multicellular organisms, such as animals (Dunning Hotopp, 2011), plants (Wickell and Li, 2020), insects (Peccoud et al., 2017), and invertebrates (Crisp et al., 2015). For example, the enzyme topoisomerase IB, typically characteristic of eukaryotes only, is found in the genome of *D. radiodurans;* knockout strains that lack this gene are more vulnerable to UV radiation than the wild type (Makarova et al., 2001). Interestingly, four *D. radiodurans* proteins with homologs in plants are associated with desiccation resistance (Makarova et al., 2001). These plant proteins belong to the late embryogenesis abundant (LEA) protein family, a well-studied group with known functions in desiccation resistance (Ingram and Bartels, 19960; Battaglia et al., 2008). It has been hypothesized that these genes also play a role in desiccation and radiation resistance in *D. radiodurans* (Mattimore and Battista, 1996).

D. radiodurans repairs its genome after oxidative stress via a two-part pathway (Cox et al., 2010). The first phase, referred to as extended synthesis-dependent single-strand annealing, involves the action of nucleases and DNA polymerases, and the second phase involves double-strand break repair mediated by RecA, a nearly universal protein with functions in both recombination and repair (Slade et al., 2009; Zahradka et al., 2006). Additionally, the pathways

utilized by *D. radiodurans* for DNA repair are considered auxiliary in *E. coli,* which uses a different primary repair pathway that does not have a homolog in *D. radiodurans* (Cox et al., 2010). The specifics of these mechanisms are not fully understood (Cox et al., 2010). However, dozens of genes and proteins have been implicated in DNA repair in *D. radiodurans* (Salmon et al., 2004). Other molecules with significant roles in DNA repair have been identified and described, including the proteins DdrA (Harris et al., 2004) and UvrA and UvrB, which are upregulated in response to desiccation in *D. radiodurans* (Ujaoney et al., 2017).

D. radiodurans exhibits significantly greater resistance to H_2O_2 than *Escherichia coli* and produces about 50 times greater catalase activity than *E. coli* after exposure to H_2O_2 (Figure 3 from Wang and Schellhorn, 1995). In addition, sublethal levels of exposure to H_2O_2 have been found to improve resistance to H_2O_2 in subsequent treatment in *D. radiodurans*, and exposed strains exhibit increased catalase production when compared to unexposed strains (Wang and Schellhorn, 1995). A similar phenomenon has been observed in *E. coli;* cells primed with 0.1 mM of H_2O_2 show significantly greater survival after being subsequently "challenged" with 1 mM of H_2O_2 than cells that are not primed (Rodríguez-Rojas et al., 2020) This indicates that the oxidative stress response may be a phenotypically plastic trait that varies with changing environmental conditions. Similar to other aerobic bacterial species, analysis of the response of *D. radiodurans* to H_2O_2 has shown that it is largely dependent on catalase activity. Strains of *D. radiodurans* with mutations in one of the three catalase genes present in its genome showed decreased resistance to H_2O_2 when compared to wild type strains (Jeong et al., 2016).

Patulibacter americanus (CP177-2)

Patulibacter americanus (type strain CP177-2) is a Gram-positive, aerobic, flagellate, motile, catalase-positive bacterium isolated from the Colorado Plateau (Reddy and Garcia-Pichel, 2009). To date, only one study has been published on this species, the study in which it was introduced (Reddy & Garcia-Pichel, 2009). *P. americanus* grows in pink colonies between 10 and 30 °C and tests positive for catalase (Reddy and Garcia-Pichel, 2009). Little is known about this organism's response or survival to oxidative stress.

Objective

Although desiccation and oxidative stress tolerance have been studied extensively, and although BSCs are known to be highly resistant to desiccation, few, if any, studies have investigated the response of BSC organisms to oxidative stress on an individual species basis. As BSCs continue to be damaged by human interference and climate change, research into their oxidative stress tolerance is warranted. A better understanding of their adaptive strategies is essential for continued efforts to prevent their destruction and promote their rehabilitation. The objective of this study is to investigate how the survival from oxidative stress induced by hydrogen peroxide in an isolate from a bacterial soil crust in the American Southwest, *Patulibacter americanus,* compares to that of *D. radiodurans.* I aim to investigate and compare the responses of *P. americanus* and *D. radiodurans* to oxidative stress by treating both *P. americanus* and *D. radiodurans* with hydrogen peroxide and determining their subsequent survival, growth, and relative amount of damage to their DNA.

MATERIALS AND METHODS

Maintenance of Cell Lines

Patulibacter americanus (CP177-2) cells were grown at room temperature on BG11-10x PGY agar media in either 60 mm x 15 mm petri dishes for experiments or 60 mm x 30 mm petri dishes for long-term storage (Table 1). Liquid cultures were grown in BG11-10x PGY broth (Table 1) in a shaking incubator at 100 rpm and at room temperature. *Deinococcus radiodurans* cells were grown at 30 °C on TGY agar media in either 60 x 15 mm petri dishes for experiments or 100 x 15 mm petri dishes for long-term storage (Table 2); petri dishes were moved to a 2 °C refrigerator after three days of growth at 30 °C for long term storage. Liquid cultures were grown in TGY broth (Table 2) at 37 °C and 200 rpm.

Procedures

Hydrogen Peroxide Treatment

Two 20 mL cultures of *D. radiodurans* labelled "treated" and "untreated" and two 20 mL cultures of *P. americanus* labelled "treated" and "untreated" were diluted to an optical density of approximately 0.1 at 600 nm (OD_{600}) using the Tecan Infinite. For each dilution, an appropriate amount of culture was extracted and moved to a fresh culture tube, and appropriate broth (TGY broth for *D. radiodurans* and BG11-10x PGY for *P. americanus*) was added to a final volume of 14.5 mL. This was reduced to a final volume of 13.9 mL after three 200 µL volumes were removed from each culture to confirm the final OD_{600} using Tecan Infinite.

Hydrogen peroxide (final concentration 30 mM H_2O_2) was pipetted into the *D. radiodurans* and *P. americanus* cultures labelled "treated." The same volume of 0.9% saline was

added to the *D. radiodurans* and *P. americanus* cultures labelled "untreated." Immediately, 1.5 mL of each culture was removed to a labelled microcentrifuge tube. Each tube was centrifuged (see centrifugation protocol, page 42). A serial dilution was performed (see dilution protocol, page 42). The contents of each dilution tube were spread plated were considered to be the "0 min exposure" cultures.

After twenty minutes of exposure, 1.5 mL of each culture were pipetted into a labelled microcentrifuge tube. Each tube was centrifuged as before and a serial dilution was performed before plating on the appropriate plates. These were considered to be the "20 min exposure" cultures.

This process was repeated every 20 minutes, with a final exposure time of 100 minutes. The entire experiment was conducted at room temperature and performed two or three times for each of the four groups (*D. radiodurans* treated, *D. radiodurans* untreated, *P. americanus* treated, and *P. americanus* untreated). See Table 3 for details regarding the dates and number of replicates of each experiment.

Recovery Growth Curves

Immediately after the 100-min time point, 200 μL of the cultures from each time point were pipetted in triplicate into two 96-well, clear, flat-bottom Tecan culture plates, one for *P. americanus* and one for *D. radiodurans.* The "broth" wells were filled with 200 μL of TGY broth for *D. radiodurans* and 200 μL of BG11-10x PGY broth for *P. americanus.* These plates were analyzed for 24 hours in Tecan Infinite (see page 43 for parameters) to collect recovery growth curves. The *P. americanus* plates were run at room temperature, and the *D. radiodurans*

plates were run at 37 °C. The remaining cultures were immediately stored in the freezer for later analysis.

DNA Extraction and PCR

DNA was extracted using the DNeasy kit from Qiagen, following manufacturer's instructions for Gram-positive bacteria. PCR was performed with 16S rRNA primers purchased from Integrated DNA Technologies (IDT), GE Healthcare illustra™ puReTaq Ready-To-Go PCR Beads, and PCR-grade water in a BioRad T100 Thermal Cycler. Genomic DNA (50 ng) was added to each PCR reaction with 12 pmol of each primer (forward and reverse), and RNase-free water was added to a final reaction volume of 25 µL (see page 43 for PCR parameters).

Gel Electrophoresis

PCR products (10 µl) were visualized on a 1% agarose gel containing SYBR Safe. Gel electrophoresis was conducted at about 95 volts for about one hour. Invitrogen DNA 1 kb Plus Ladder was used as a standard. Images of gels were taken with a Bio-Rad ChemiDoc MP Imaging System.

Data Analysis

Survival Analysis

After the completion of each experiment, the average number of colony forming units (CFUs) per mL in each group (treated/untreated, time point) were calculated from plates. Colonies were counted after three days of growth for *D. radiodurans* and after five days of growth for *P. americanus.* For each exposure time past 0 min, the number of CFUs per mL was

divided by the number of CFUs per mL at 0 min and multiplied by 100 to determine percent survival.

Growth Curve Analysis

Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (Elso et al., 2004). The tool "computes a permutation p-value for each pair of groups, using the average t-statistic between the groups as the test statistic. t-tests are computed for each time and averaged to obtain the permutation statistic" (Smyth et al., 2019). The tool is a web interface, and the actual calculations are performed by the compareGrowthCurves function, which is part of the statmod package in R. This test determines whether two growth curves are statistically different in either shape or magnitude. The growth of treated and untreated cultures after each time point of H_2O_2 treatment were compared to one another to determine significant differences in growth; 100 permutations were utilized for each comparison. The tool was located at this link as of April 22, 2021: http://bioinf.wehi.edu.au/software/compareCurves/

RESULTS

Survival after H_2O_2 Treatment

D. radiodurans

Three independent experiments were performed, and the average survival percentages after exposure to either H_2O_2 or 0.9% saline were recorded. *D. radiodurans* cultures treated with H_2O_2 exhibited a steady, near-linear decline in percent survival as a function of increased duration of H_2O_2 exposure (Figure 4). After 100-min of exposure to H_2O_2, viability was reduced

to about 14%. In contrast, untreated *D. radiodurans* cultures exposed to 0.9% saline exhibited variations in survival, alternating increasing and decreasing survival with each 20-min time interval until returning close to 0-min baseline (about 100%) after 100 min of exposure (Figure 4).

P. americanus

From 0-60 min of exposure, treated and untreated *P. americanus* cultures showed similar patterns of survival (Figure 5). Both treated and untreated cultures increased between 0 and 20 min to about 140% and 160%, respectively, decreased between 20 min and 40 min to about 120% and 110%, and increased between 40 and 60 min to about 150% and 140%. Between 60- and 80-min, however, treated cultures increased slightly, while untreated cultures dropped to about 130%. Between 80- and 100-min, treated cultures continued to exhibit growth. After 100 minutes of exposure, treated cultures grew to about 190% of the number of colonies present at 0 minutes of exposure. In contrast, untreated cultures continued to decline, with about 110% of cells surviving after 100 min of exposure to 0.9% saline.

Growth Curves After Removal of Treatment

D. radiodurans

D. radiodurans strains exposed to H_2O_2 exhibited noticeably less growth after removal of treatment when compared to unexposed strains (Figures 6-8). This difference was significant (p-value <0.01) at the 40-, 60-, 80-, and 100-min time points (Figure 8, Table 4). However, none of the growth curves of any of the treated cultures were significantly different from each other (Figure 6, Table 5). Similarly, there were no significant differences between the growth curves of

any untreated cultures (Figure 7, Table 6). The growth curves of all replicates were extremely variable.

P. americanus

Growth (measured as OD_{600}) of *P. americanus* cultures after treatment removal was comparable to growth of untreated *P. americanus* cultures for all exposure times (Figures 9-11). There was no statistically significant (p-value <0.01) difference between treated and untreated cultures at any of the exposure time points, indicating that the growth curves for all groups were comparable (Figure 11, Table 7). Additionally, there were no significant differences between any of the treated growth curves (Figure 9, Table 8), nor between any of the untreated growth curves (Figure 10, Table 9), indicating that these curves were also comparable.

Gel Electrophoresis

D. radiodurans

All PCR products were about 1,500 base pairs in size, which is consistent with the size of 16S rRNA genes (Clarridge, 2004). PCR products of untreated *D. radiodurans* strains exhibited bands of similar brightness at each exposure time, with the exception of the first replicate, which was more variable; bands were brighter at 20-, 60-, and 80-min exposure when compared to 0-, 40-, and 100-min exposure for this replicate (Figure 12; original images shown in Figures 14-17). PCR products of treated *D. radiodurans* strains showed a decreasing gradient of band brightness, with 0 min exposure strains exhibiting the brightest bands, and 100 min exposure strains exhibiting the faintest bands, with the exception of the second replicate, which showed the brightest bands at 0-, 80-, and 100-min exposure and fainter bands at 20-, 40, and 60-min

exposure (Figure 12). All PCR products displayed a very faint band around 100 bp, indicating the presence of "primer dimers."

P. americanus

All PCR products were about 1,500 base pairs in size, which is consistent with the size of 16S rRNA genes (Clarridge, 2004). PCR products of untreated *P. americanus* strains exhibited bands of similar brightness at each exposure time; this was consistent across all three replicates (Figure 13; original images shown in Figures 14-17). PCR products of treated *P. americanus* cultures of the first and third replicates showed an increasing gradient of band brightness up to 60-min exposure, and then a decreasing gradient from 60- to 100-min exposure (Figure 13). PCR products of treated *P. americanus* strains of the second replicate showed an increase in band brightness from 0- to 20-min of exposure, followed by a decreasing gradient, although the 100-min exposure did not exhibit a band for this replicate (Figure 13). All PCR products displayed a very faint band around 100 bp, indicating the presence of "primer dimers."

DISCUSSION

Survival After H_2O_2 Treatment

Survival of *D. radiodurans* cultures exposed to H_2O_2 declined with increasing exposure time, with a final survival of 14.2% after 100 min of exposure. Unexposed cultures showed about 103% survival after the same amount of time, indicating slight growth. In contrast, *P. americanus* exhibited far greater survival after exposure to H_2O_2 than *D. radiodurans.* In fact, *P. americanus* cultures exposed to H_2O_2 grew at a faster rate than unexposed cultures; cultures exposed for 100 minutes exhibited about 190% survival when compared to cultures exposed for

0 minutes, the number of cells nearly doubling. This value that far exceeds that of the untreated *P. americanus* cultures (107%). These results suggest that *P. americanus* is more resistant to H_2O_2 than *D. radiodurans.* This is surprising, considering that *D. radiodurans* is known to be exceptionally resistant to H_2O_2 compared to other bacteria (Boling and Setlow, 1966).

Additionally, these results suggest that *P. americanus* is capable of continued growth in the presence of H_2O_2, and that its growth rate under these conditions exceeds that of its normal growth rate. Considering the multiple mechanisms by which ROS cause damage to cellular biomolecules, including DNA (Girard and Boiteux, 1997), proteins, and lipids (Auten and Davis, 2009), it is surprising that *P. americanus* was able to maintain normal or even elevated growth rates during oxidative stress. Although *P. americanus* is catalase-positive, the response of *D. radiodurans* to H_2O_2 is known to be largely dependent on catalase activity, and *D. radiodurans* exhibited decreased survival with increasing exposure time in this experiment. Replication of genetic material occurs prior to cell splitting in binary fission (Bartee and Brook, 2019), so it would be expected that, after an oxidative stressor was introduced to a culture, replication and growth would halt while DNA and other molecules were repaired, and that this growth cessation would occur for the duration of oxidative stress. However, based on survival and growth, *P. americanus* appears to either repair damage at a faster rate than it is caused by oxidative stress or process oxidative stressors with enough efficiency to prevent damage altogether. Alternatively, the initial stress could cause *P. americanus* to increase its expression of catalase, which could then reach a level that is sufficient to eliminate the stress as it occurs.

The increase in growth of treated cultures when compared to untreated cultures in *P. americanus* is another unexpected finding. Although it has been shown that priming both *D. radiodurans* and *E. coli* with a low dose of H_2O_2 leads to increased resistance to H_2O_2 after a

subsequent higher dose (Wang and Schellhorn, 1995; Rodríguez-Rojas et al., 2020), a growth advantage has not been documented either during or after treatment with H_2O_2.

In addition, there was significant variation in the survival of untreated cultures of both *D. radiodurans* and *P. americanus,* as well as in treated cultures of *P. americanus,* with alternating increases and decreases in survival seen between each time point. Although this pattern was consistent across replicates, it is important to note that only two replicates of the *P. americanus* survival experiment were performed for treated cultures, compared to the three replicates of the *D. radiodurans* experiment, and that untreated *P. americanus* cultures have either two or three replicates depending on the time point (see Materials and Methods, Table 3). This investigation may be limited by the small number of replicates. In addition, CFUs were counted after five days of growth on plates for *P. americanus,* which was generally the first day that colonies were observed to appear. In contrast, *D. radiodurans* CFUs were counted after three days of growth, but colonies were usually first observed on the second day of growth. Waiting until the sixth day of growth for *P. americanus* may have resulted in a more consistent measurement of growth across the two species. These aspects of the study design may have contributed to the variation in growth seen here; however, the general alternating pattern of growth and decline between time points exhibited by these cultures was fairly consistent across replicates.

It is important to reiterate here that the untreated cultures were exposed to 0.9% saline rather than water. Although H_2O_2 is suspended in water, saline was used to avoid a change in osmolarity that would affect results. It is possible that the added saline may have affected the growth of the untreated cultures of *D. radiodurans* or *P. americanus.* However, the final concentration of NaCl in the untreated cultures was about 0.03%. This is most likely a negligible

amount that would not affect the growth of either organism, but this cannot be known with complete certainty based on this experiment alone.

Growth Curves After Removal of Treatment

Statistical significance of growth curves was determined using the CGGC permutation test described earlier (page 15), and 100 permutations were used. The authors suggest 10,000 permutations for definitive results, but due to the large number of time points in each growth curve and the number of replicates, the tool was not able to process any more than 100 permutations. Because of this, the p-values listed in this study may not be accurate. Future students would benefit from growth curves with fewer times points in order to obtain more accurate statistical significance of their results.

Treated and untreated *P. americanus* cultures exhibited extremely similar growth curves after removal of treatment at all time points (Figure 11), with no significant differences at any of the time points (Table 8). This indicates that H_2O_2 did not impact the cultures to an extent that restricted growth after cessation of treatment. Considering the increased growth rate seen in treated *P. americanus* cultures (Figure 5), it is interesting that this rate was not maintained after treatment was removed via centrifugation. This would suggest that oxidative stress facilitates growth initially, but that this benefit only occurs *during* the time of stress and not afterwards.

Treated and untreated *D. radiodurans* growth curves after removal of treatment were significantly different after 40-, 60-, 80-, and 100-min exposure, although these results may be inaccurate. All culture plates containing *D. radiodurans* cultures were covered with a lid during optical density analysis in order to prevent drying out since the Tecan instrument was maintained at 37 °C; it had been observed previously that growth curves conducted without a lid resulted in

dried out cultures and inaccurate readings. However, it was determined from this experiment that condensation formed on the lid of culture plates, also presumably as a result of the 37 °C temperature, since this condensation did not form in *P. americanus* plates. The resulting growth curves were extremely variable across and within all three replicate experiments for *D. radiodurans,* and inaccuracies due to the observed condensation are suspected. Therefore, differences in growth curves between treated and untreated *D. radiodurans* cultures may not be due to the experimental conditions, but to the inaccurate data collection, and should be analyzed carefully. If accurate, these results would indicate that the growth of *D. radiodurans* was significantly inhibited by H_2O_2, although there was no gradient seen in this effect; the degree of inhibition did not increase with increasing exposure time, and there were no statistically significant differences between the treated cultures.

Future students should attempt to measure a growth curve using a different mechanism in order to verify this potential finding. The condensation on the culture plate lid blocks measurement of optical density, but the lid cannot be removed without causing complete desiccation of the cultures, resulting in inaccurate growth curve data. To account for this, students could measure a growth curve at room temperature rather than at 37 °C to avoid the formation of condensation, keeping in mind that *D. radiodurans* exhibits optimal growth at 37 °C, and that the lower temperature may affect results (He, 2009).

Alternatively, periodic extraction and measurement of *D. radiodurans* liquid cultures could be utilized to measure growth curves instead. The culture would be grown under normal conditions for *D. radiodurans* (see Materials and Methods) and OD_{600} would be measured at regular time points. However, the user manual for Tecan Infinite M Plex suggests that no less than a third of the maximum volume of each well in the culture plate be used in order for

accurate optical density measurement. The maximum volume of wells in 96-well culture plates is about 0.36 mL, so a minimum volume for optical density reading would be about 120 μL. Measurements are usually taken in triplicate, so 360 μL of sample would need to be obtained at each growth curve time point. Depending on how many time points were used to create the growth curve and considering that six samples at six different exposure times were taken from the initial treated and untreated cultures for this experiment, very large initial cultures would be needed to create these growth curves, as well as larger samples taken at each exposure time. Centrifugation of these larger samples would require different equipment than the microcentrifuge used in this experiment. Cessation of H_2O_2 treatment could be performed via application of catalase, similar to the methods described by Yang et al. (2016), rather than centrifugation, but there is significant potential for increased variation due to larger culture size that may affect results.

Gel Electrophoresis

Gel electrophoresis of PCR products showed variation in amplification of the housekeeping gene coding 16S rRNA across treated and untreated samples of *D. radiodurans* and *P. americanus.* PCR involves the denaturation, annealing, and extension of a segment of DNA complementary to the primer pairs. DNA polymerase can only replicate the DNA segment if it is intact with no lesions. Therefore, decreased amplification of PCR product may indicate a greater number of DNA lesions (Grimaldi et al. 2002). The decreased amplification can be attributed to DNA lesions rather than variations in quantity of DNA because the same amount of genomic DNA (50 ng) was added to each PCR reaction. This is visualized as brighter bands

(more amplification) or fainter bands (less amplification) on an agarose gel; the same volume of PCR product was added to each lane of the gel.

Two out of three replicates of *D. radiodurans* showed a decreasing gradient of band brightness as length of exposure to H_2O_2 increased. This implies that *D. radiodurans* sustained DNA damage from H_2O_2, and that the amount of damage increased over time. Two out of three replicates showed consistent brightness of untreated *D. radiodurans* bands, indicating a consistent number of lesions at each exposure time. However, the third replicate of each of these groups showed more variability. *P. americanus* also exhibited consistent band brightness across untreated strain PCR products, also indicating a consistent number of lesions. Treated cultures showed an increasing gradient of band brightness up to 60-min exposure, followed by a decreasing gradient, in two of three replicates, indicating that the number of lesions decreased with increasing exposure time up until 60 minutes of exposure, at which point the number of lesions increased. The third replicate showed an increase in brightness from 0- to 20-min exposure, followed by a decrease up to 80-min exposure, with the 100-min exposure showing no band, potentially as a result of pipetting error or other error.

DNA damage in *D. radiodurans* was expected; it has been shown that *D. radiodurans* sustains a similar amount of damage to its DNA following oxidative stress when compared to other bacteria, and that its resistance to oxidative stress is a result of its DNA repair capabilities rather than the prevention of damage (Krisko and Radman, 2013). The decrease in lesions over time in *P. americanus* was an unexpected finding. This result could indicate that *P. americanus* has an extremely efficient DNA repair ability that outpaces that of *D. radiodurans,* allowing it to recover from DNA damage while oxidative stress is occurring. Interestingly, this repair system appears to be limited; lesions decreased between 0- and 60-min of exposure, but increased again

afterwards. This could indicate that exposure to H_2O_2 triggers a DNA damage repair pathway that is initially effective in repairing DNA, but eventually is overwhelmed as oxidative stress persists. However, the pattern of DNA damage in *P. americanus* was not reflected in its survival. Based on the decreasing and then increasing number of DNA lesions seen after PCR, I would have expected to see consistent or increasing survival as DNA is repaired, followed by a decrease as the number of lesions increased after 60 min of exposure. Instead, *P. americanus* showed the most consistent increase in growth after 60 min of exposure. Further investigation is needed to confirm this disparity and understand its biological basis.

D. radiodurans does not appear to have the ability to repair its DNA while oxidative stress is occurring based on these results, since increasing damage was seen as exposure to H_2O_2 increased. However, it has been shown that *D. radiodurans* can repair its genome over the course of several days after an acute exposure to oxidative stress-causing ionizing radiation (Harris et al. 2004). It is possible that *D. radiodurans* uses a similar long-term DNA damage repair process after exposure to H_2O_2 that results in greater restoration of genome integrity, while *P. americanus* uses a faster, but ultimately shorter-lived, repair mechanism. If this is the case, the DNA repair system of *D. radiodurans* may be a more effective adaptation in the long term, or in times of prolonged exposure to oxidative stress.

However, there was significant variation seen in the results for both *D. radiodurans* and *P. americanus.* This variability may be attributable to pipetting error, but could indicate heterogeneity in response to oxidative stress within individuals of each species. Further research should use qPCR to quantify the amount of damage sustained by *D. radiodurans* and *P. americanus* strains after each exposure time, following a methodology similar to that of Zhu and Coffman (2017). This could more accurately identify the effects of H_2O_2 exposure on DNA

damage, and identify more consistent patterns in repair over time. qPCR could also be used to identify the number of lesions after treatment is removed, to determine how DNA is repaired by *D. radiodurans* and *P. americanus* after oxidative stress has ended. In addition, PCR and qPCR using different primers may be a useful avenue of future study. 16S rRNA genes were chosen for this experiment because they are highly conserved across bacterial species, but it is possible that their location within the genome could influence the amount of damage that they sustain. A different, equally conserved gene could be used to confirm these findings.

Summary and Future Directions

Overall, the results suggest that *P. americanus* is highly resistant to oxidative stress induced by H_2O_2, potentially more so than *D. radiodurans.* One potential explanation for the increased resistance of *P. americanus* to H_2O_2 is a more impervious cell membrane or cell wall that does not allow for diffusion of H_2O_2 into the cytoplasm. Although both *D. radiodurans* and *P. americanus* are Gram positive-staining bacteria (Reddy and Garcia-Pichel, 2009; Minton, 1994), *D. radiodurans* exhibits a unique structure. Its membrane is surrounded by an outer envelope of proteins and glycoproteins called the Surface layer, or S-layer, which is found in some microorganisms (Farci et al., 2014). The S-layer of *D. radiodurans* is unique in that it is integrated into a larger structure outside of the cell membrane with multiple interconnected layers, referred to as the pink envelope (Farci et al., 2014). Additionally, it contains some similarities to Gram negative cell wall structures, thought to be vestigial from an ancestral species (Farci et al., 2014). Although the membrane of *P. americanus* has not been studied directly, it has been found to be Gram positive (Reddy and Garcia-Pichel, 2009). Gram positive bacteria exhibit thick layers of peptidoglycan in their cell walls (Pasquina-Lemonche et al.,

2020). Both structures are adapted for protection and defense, but achieve this in different ways. If the *P. americanus* cells are more impervious to H_2O_2 than *D. radiodurans*, this could explain the difference in survival and DNA damage between the two organisms. In fact, it has been found that membrane permeability may be a defense mechanism employed by bacteria against the damaging effects of H_2O_2, although the magnitude of this effect varies depending on the concentration of H_2O_2 present (Uhl and Dukan, 2016).

However, it has been suggested that H_2O_2 can enter the cell via aquaporins in plants and mammals (Bienert et al., 2006). Since aquaporins are highly conserved across the kingdoms of life (Kruse et al., 2006), H_2O_2 may also diffuse across the membrane using aquaporins in bacteria. Furthermore, when H_2O_2 was added to the *D. radiodurans* and *P. americanus* cultures in this experiment, the production of visible bubbles occurred immediately and appeared to be approximately equal. Bubbling after treatment with H_2O_2 indicates the activity of catalase (Reiner, 2010). In theory, if less H_2O_2 diffused across the *P. americanus* membrane compared to the *D. radiodurans* membrane, less or no bubbles would be observed in the *P. americanus* cultures. However, this visible assessment is extremely subjective. Since catalase is known to be involved in the response of *D. radiodurans* to H_2O_2 (Jeong et al., 2016), it would be beneficial for future studies to analyze the production of this enzyme in *P. americanus* cultures compared to *D. radiodurans* cultures before and after exposure to H_2O_2. Further research could utilize RT-qPCR or Western Blot analysis to determine variations in the production of catalase in response to H_2O_2, giving a more quantitative and direct insight into this potential phenomenon.

Another potential explanation is that *P. americanus* responds to and/or removes oxidative stressors more efficiently than *D. radiodurans*, either by neutralizing free radicals very quickly after their generation or preventing the formation of free radicals by converting H_2O_2 to oxygen

and water at a faster rate than *D. radiodurans* via upregulation of catalase production. To my knowledge, there is no previously studied example of a bacterium capable of this kind of resistance. Similarly, the absence of DNA damage in *P. americanus* strains suggest that *P. americanus* has a faster, more efficient, or more effective DNA damage repair system than *D. radiodurans.*

A wide variety of paths for future research are opened by this potential finding. There are a plethora of biomolecules and pathway implicated in oxidative stress resistance and repair in *D. radiodurans* and other organisms. Investigations into their involvement in *P. americanus* could explain its potential resistance to oxidative stress. For example, a variety of proteins are known to be involved in DNA damage repair in *D. radiodurans,* including the proteins DNA damage response A (DdrA) and RecA, antibodies against which were obtained from Dr. Cox at the University of Wisconsin and Dr. Bishop at the University of Chicago, respectively. RecA is a DNA-dependent ATPase with a well-documented role in stress response and DNA damage repair (Cox, 2017). RecA is central to homologous recombination, but also plays important roles in induction of the SOS response and repair of double-strand DNA breaks (Cox, 2003); in fact, it has been theorized that RecA evolved for the purpose of DNA repair, and that its effects on genetic diversity through homologous recombination are a secondary benefit (Cox, 1993). RecA is essential for the expression of the remarkable radiation resistance phenotype that characterizes *Deinococcus radiodurans* (Minton, 1996). DdrA, a less well-studied protein isolated from *D. radiodurans*, was found to significantly improve the preservation of genomic DNA shortly after irradiation, with a more than 3-fold effect (Harris et al., 2004, p. 1632-3), and *D. radiodurans* double *recA* and *ddrA* mutants are more vulnerable to radiation than single mutants of either gene (Harris et al., 2004), indicating an essential role in DNA damage recovery. Although

protein analysis could not be performed for this experiment due to time constraints related to both the scope of the project and the COVID-19 pandemic, future students now have access to both antibodies, and could use them to study changes in DdrA and RecA production as a result of H_2O_2 exposure via Western Blot experiments.

CONCLUSIONS

Overall, the increased survival after exposure to H_2O_2, the similarity in growth of treated and untreated cultures, and the decreased or absent DNA damage after exposure to H_2O_2 suggest that *P. americanus* exhibits remarkable resistance to oxidative stress and/or DNA damage repair capabilities, greater than that of *D. radiodurans*, a highly oxidative stress-resistant organism. As an isolate of a BSC, *P. americanus* is frequently subjected to desiccation in its natural environment and may have developed oxidative stress resistance and recovery mechanisms that equal or surpass those of *D. radiodurans* as a mechanism of survival. Future research should be conducted in order to determine whether this resistance applies to other forms of oxidative stress, such as UV and ionizing radiation. Additionally, other BSC isolates could be studied in a similar manner to determine whether this level of resistance is consistent across other species. In light of changing climate conditions and increasing anthropogenic damage to BSCs, understanding the stress responses of BSC isolates is an extremely important area of research. This investigation provides a foundation for future studies that will elucidate BSC isolate oxidative stress resistance abilities and mechanisms, facilitating the study and rehabilitation of BSCs moving forward.

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APPENDIX

Protocols

Removal of H_2O_2 or 0.9% Saline by Centrifugation

1.5 mL of culture was centrifuged for 5 min at 10,000 x g at room temperature to pellet the cells. The supernatant was discarded and replaced with 1.5 mL of the appropriate broth (TGY broth for *D. radiodurans* and BG11-10x PGY for *P. americanus*). The mixture was vortexed to resuspend the cells. The microcentrifuge tube was then centrifuged once again for 5 min at 10,000 x g at room temperature. This procedure was repeated once, followed by resuspension in the appropriate growth media.

Serial Dilution

10 μL of culture was pipetted into a microcentrifuge containing 100 μL of sterile saline and labelled 10^{-1}. This was repeated to 10^{-5} for *D. radiodurans* and 10^{-7} for *P. americanus*. However, this is not a 10-fold dilution due to the final volume of 110 μL instead of 100 μL. This has been corrected in the calculations.

Equipment

All OD_{600} and growth curve data were taken using Tecan Infinite M Plex or Tecan Infinite M200 Pro and the iControl software, using 96-well clear flat-bottom plates purchased from Tecan. All centrifugations were completed in a Sorvall Legend Micro 21R Centrifuge. Concentrations of DNA were measured using an Eppendorf Basic BioSpectrometer. All PCR was conducted using a BioRad T100 Thermal Cycler.

Equipment Programming

Tecan

Measurements of OD_{600} were made using the following parameters: Absorbance (600 nm, 25 flashes, 0 ms settle time).

Growth curves for *D. radiodurans* were measured using the parameters shown in Table 10, modified from the "Automated Solution for Monitoring Growth of *Staphylococcus aureus*" application notes for Infinite 200 Pro.

Table 10: Parameters used to measure OD_{600} growth curves for *D. radiodurans* and *P. americanus.*

Parameter	***D. radiodurans***	***P. americanus***
Kinetic Cycle	23:59:00	23:59:00
Kinetic Interval	15 min	14 min
Absorbance	600 nm, 25 flashes, 100 ms settle time	600 nm, 25 flashes, 100 ms settle time
Incubation	Shaking, orbital, 3.5 mm, 800 seconds	Shaking, orbital, 6 mm, 800 seconds
Temperature	37 °C	n/a
Wait for Temperature	36.5 °C to 37.5 °C	n/a
Equipment Used	Tecan Infinite M200 Pro	Tecan Infinite M Plex

PCR

All PCR analyses were run with the following parameters:

-Cycle 1: 1 repeat, 05:00 min, 95.0°C

-Cycle 2: 30 repeats

Step 1 (denature): 00:15 min, 95.0 °C

Step 2 (anneal): 00:30 min, 55.0 °C

Step 3 (extend): 01:00 min, 72 °C

-Cycle 3: 1 repeat, 05:00 min, 72 °C

-Cycle 4: 12 °C, infinite hold

TABLES AND FIGURES

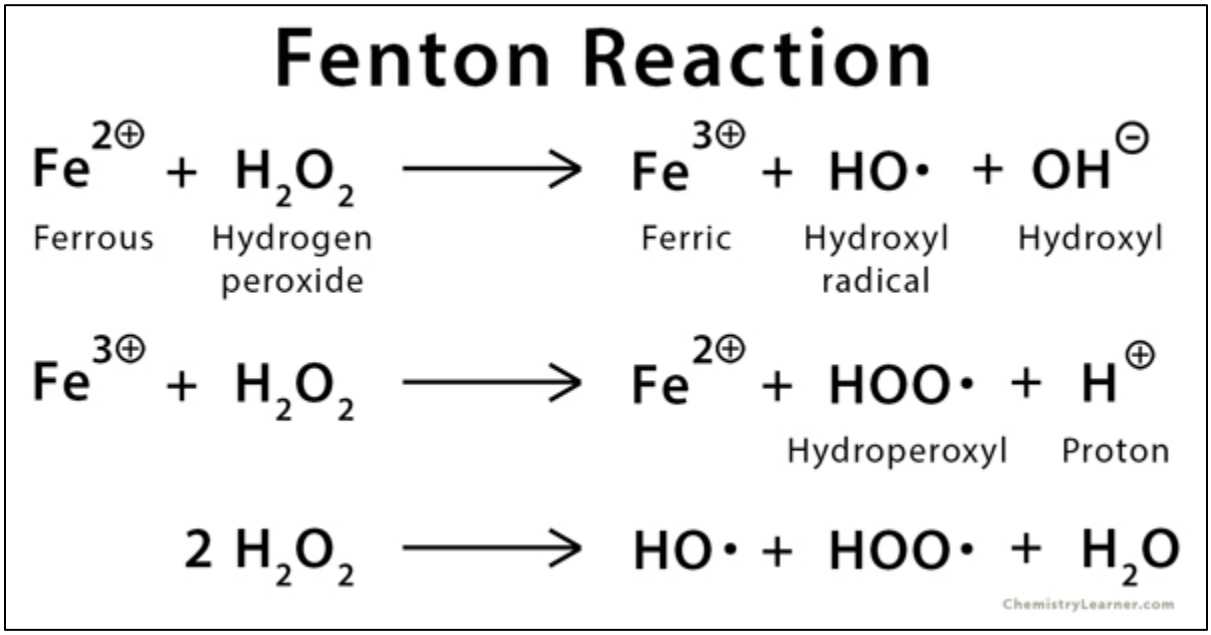

Figure 1: Variations of the Fenton Reaction, the conversion of hydrogen peroxide to a hydroxyl radical and a hydroxyl ion, catalyzed by ions of iron. Image from https://www.chemistrylearner.com/fenton-reaction.html.

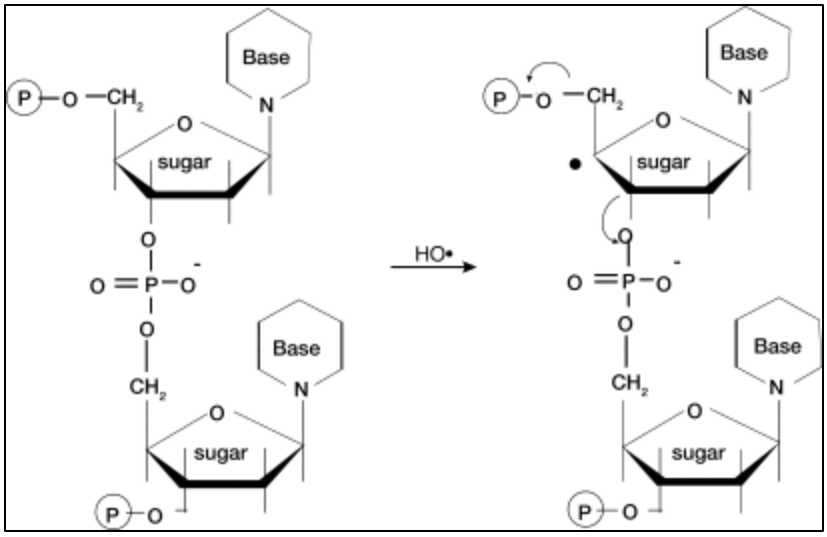

Figure 2: Oxidation of nucleic acids by a hydroxyl radical, leading to single-strand breaks at the phosphodiester bond. Image from Clark, 2008.

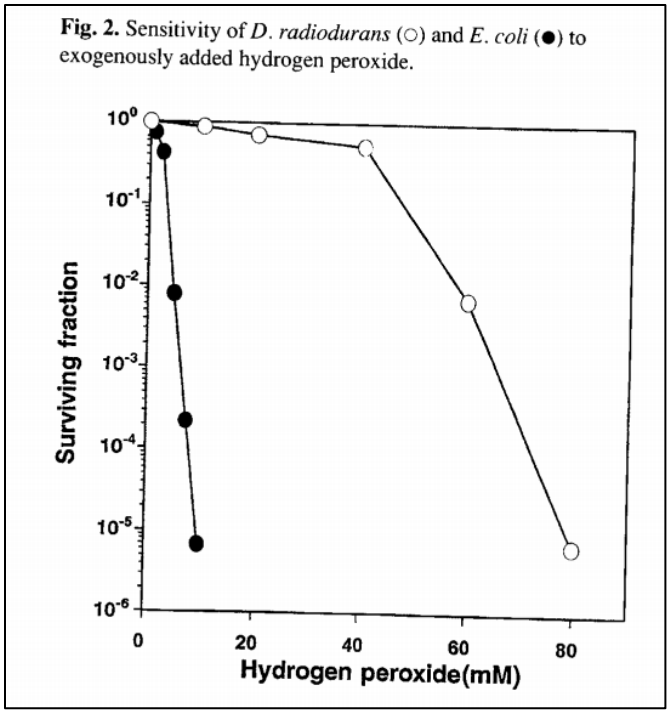

Figure 3: Survival of *D. radiodurans* and *E. coli* after 30 minutes of exposure to H_2O_2 at varying concentrations. From Wang and Schellhorn, 1995.

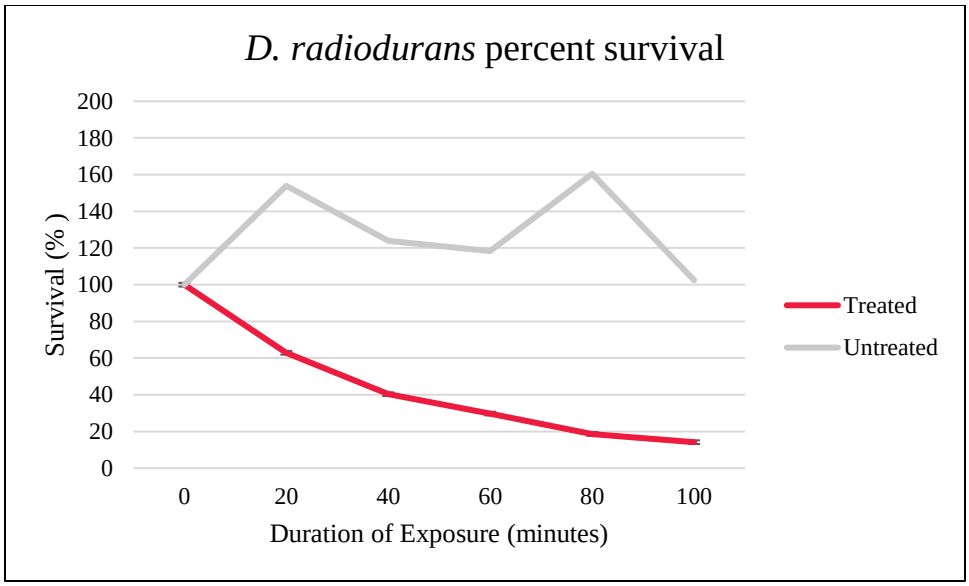

Figure 4: Survival of *D. radiodurans* cultures following exposure for 0, 20, 40, 60, 80, and 100 minutes. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. Survival indicates the average of three experiments.

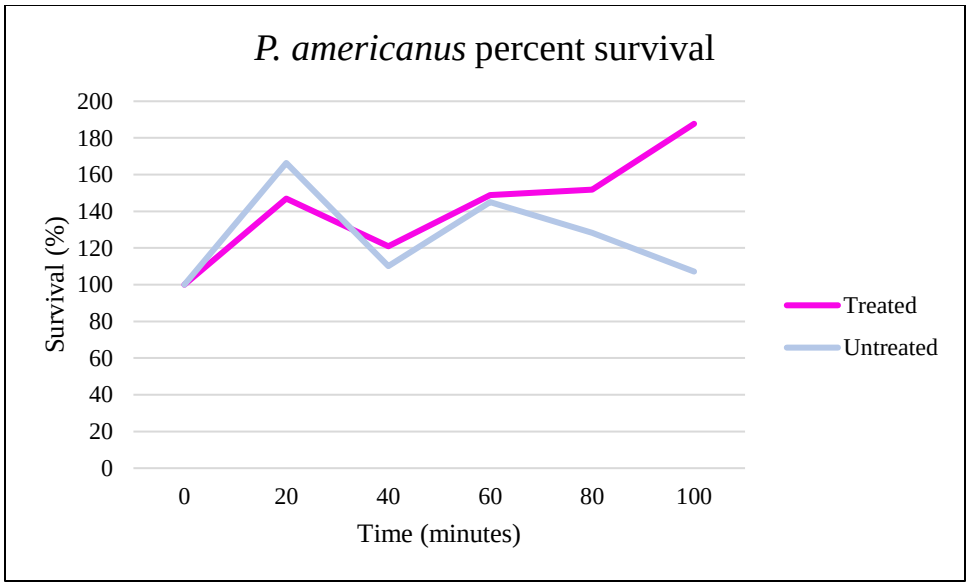

Figure 5: Survival of *P. americanus* cultures following exposure for 0, 20, 40, 60, 80, and 100 minutes. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. Survival indicates the average of two experiments for treated cultures and for 20, 60, and 80 min time points for untreated cultures, and the average of three experiments for 0, 40, and 100 minute time points of untreated cultures (see Table 3).

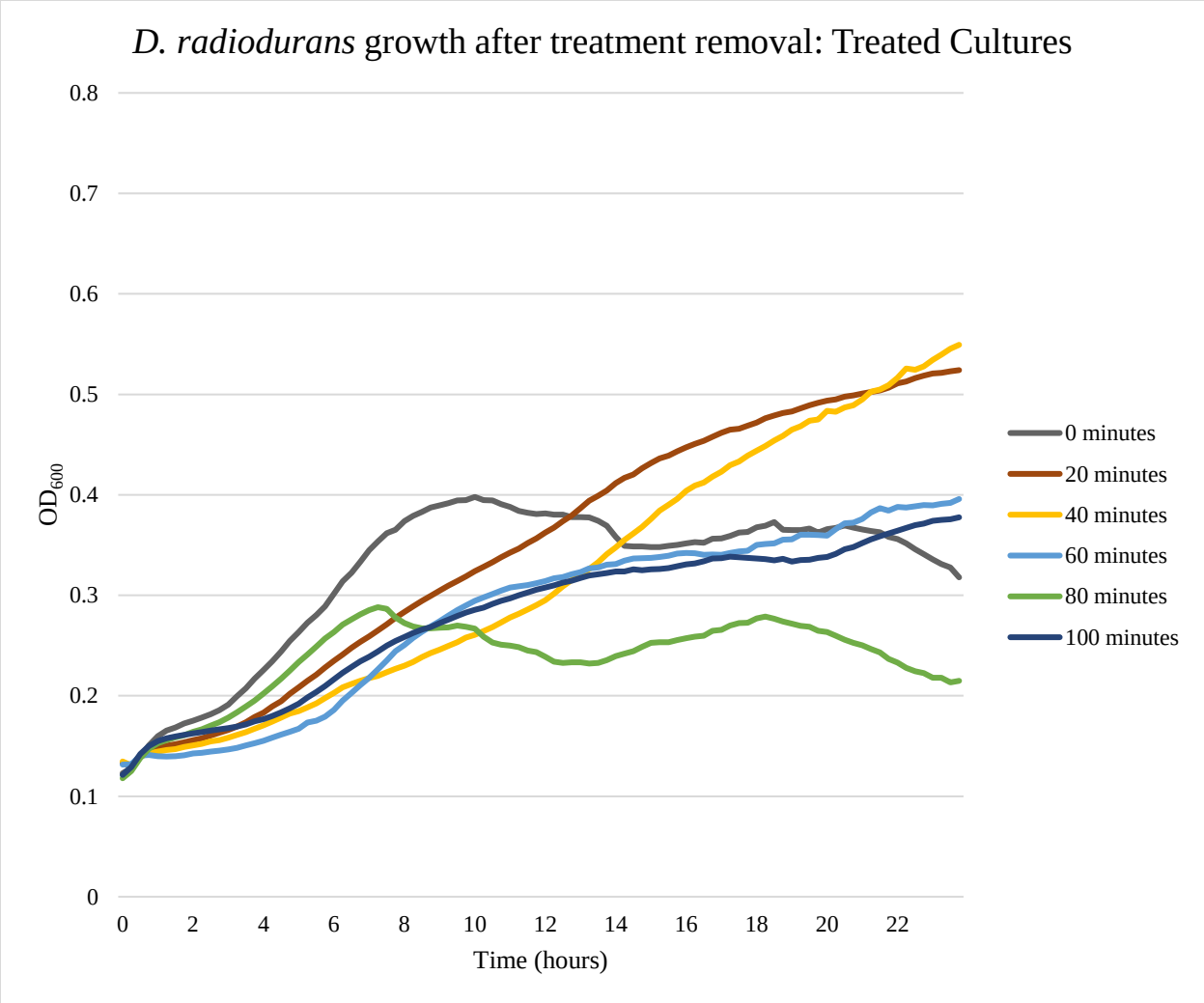

Figure 6: Recovery growth curves of *D. radiodurans* cultures after exposure to H_2O_2. H_2O_2 was removed via centrifugation prior to growth curve analysis. Each curve indicates the average of three replicates from of three experiments, for a total of nine replicates (see Table 3).

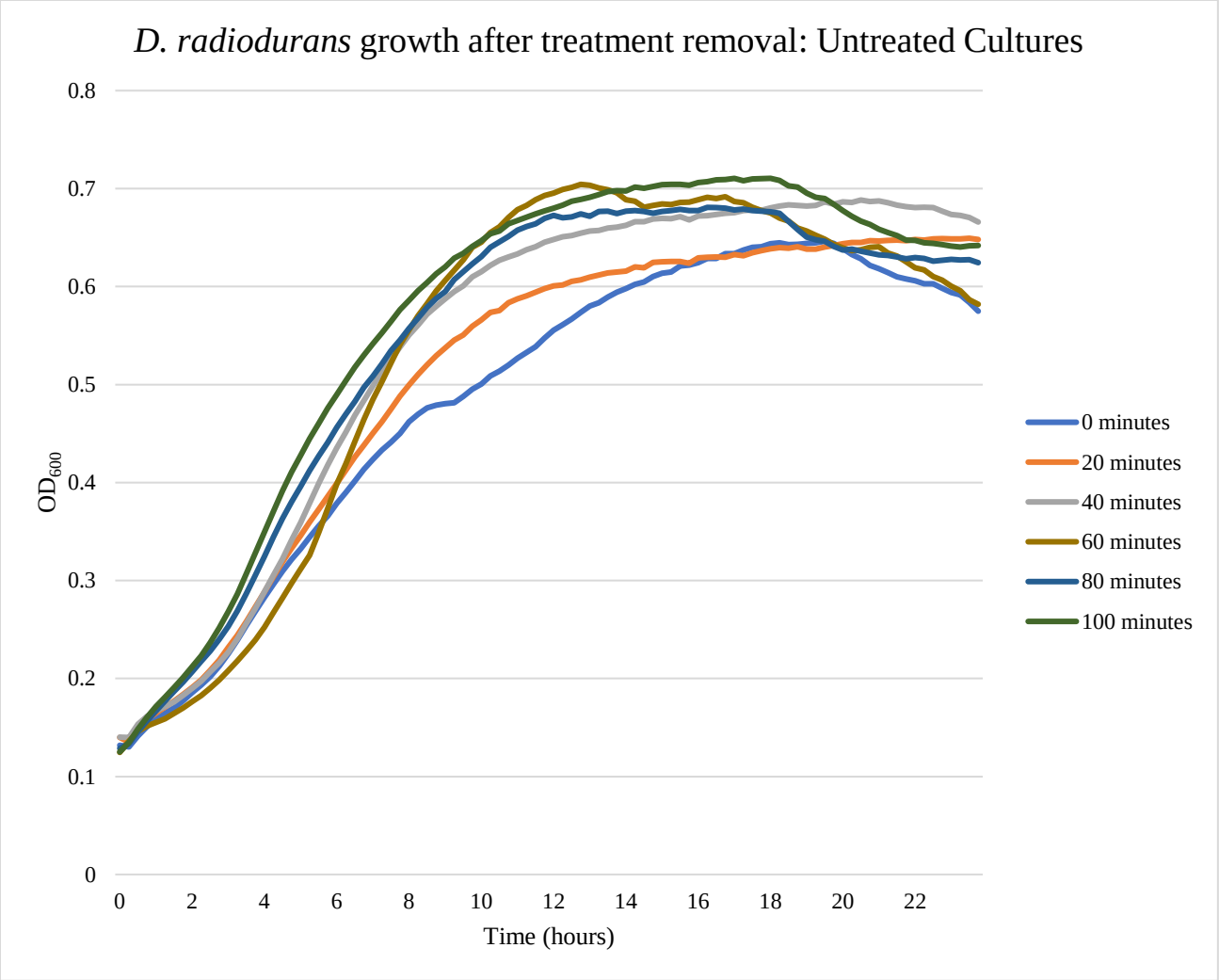

Figure 7: Recovery growth curve of *D. radiodurans* cultures after exposure to 0.9% saline. Saline was removed via centrifugation prior to growth curve analysis. Each curve indicates the average of three replicates from three experiments for a total of nine replicates (see Table 3).

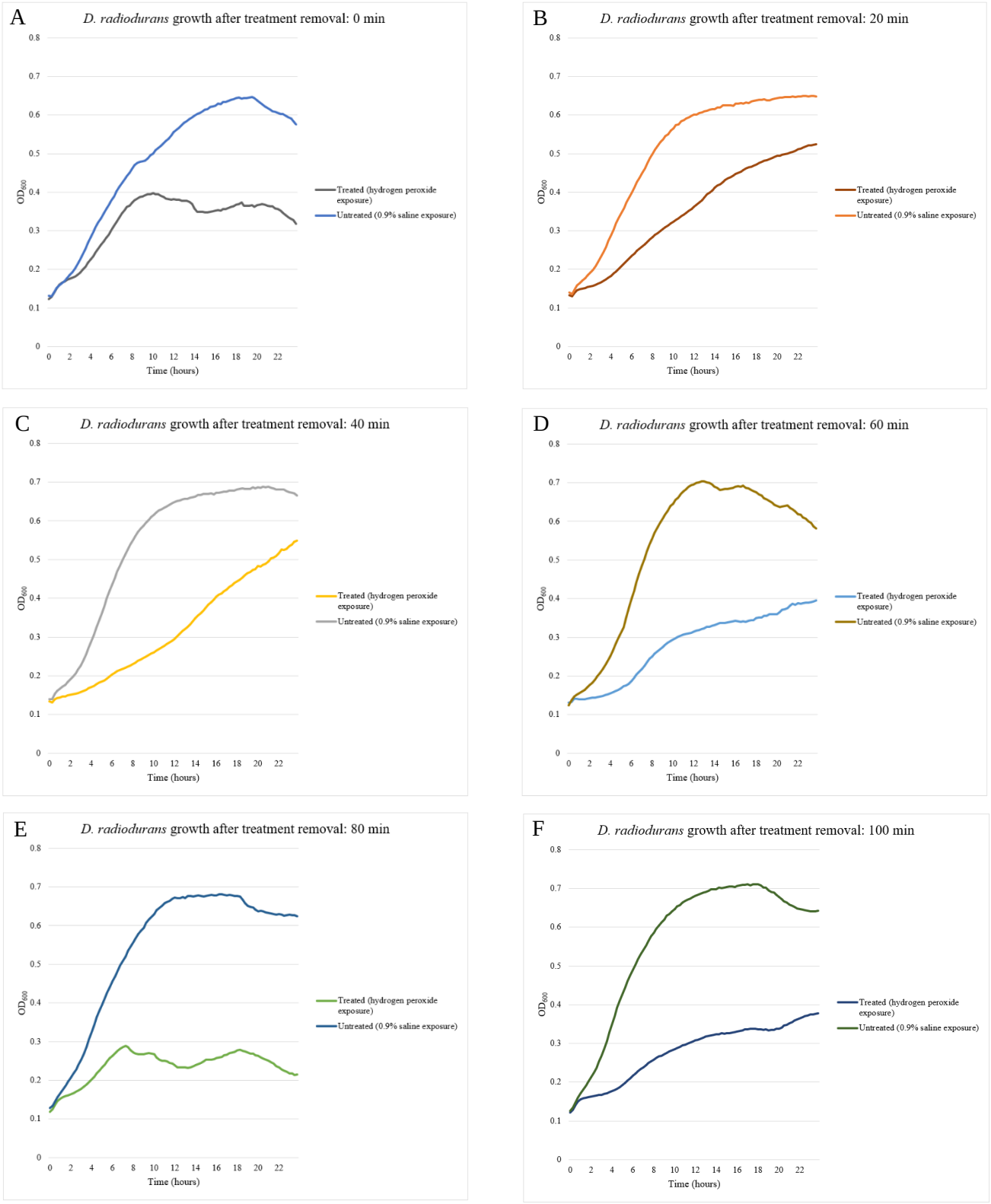

Figure 8: Recovery growth curve of *D. radiodurans* cultures after (A) 0, (B) 20, (C) 40, (D) 60, (E) 80, and (F) 100 minutes of exposure to H_2O_2 (Treated) or 0.9% saline (Untreated). H_2O_2 or 0.9% saline was removed via centrifugation prior to growth curve analysis. Each curve indicates the average of three replicates from three experiments for a total of nine replicates (see Table 3).

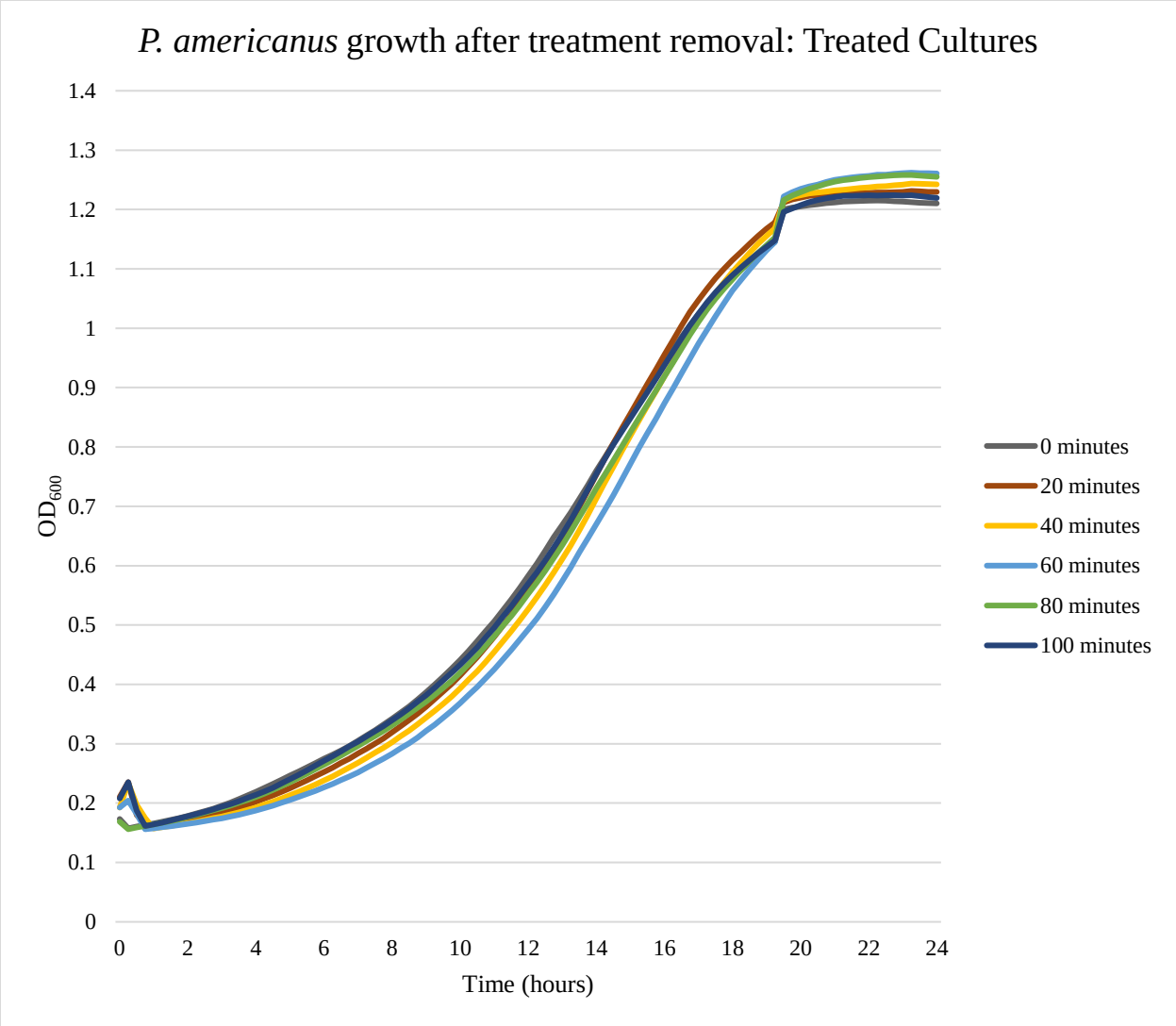

Figure 9: Recovery growth curve of *P. americanus* cultures after exposure to H_2O_2. H_2O_2 was removed via centrifugation prior to growth curve analysis. Each curve indicates the average of three replicates from three experiments for a total of nine replicates up to 19 hours and 15 minutes; afterwards, the curves indicate the average of three replicates from two experiments for a total of six replicates (see Table 3).

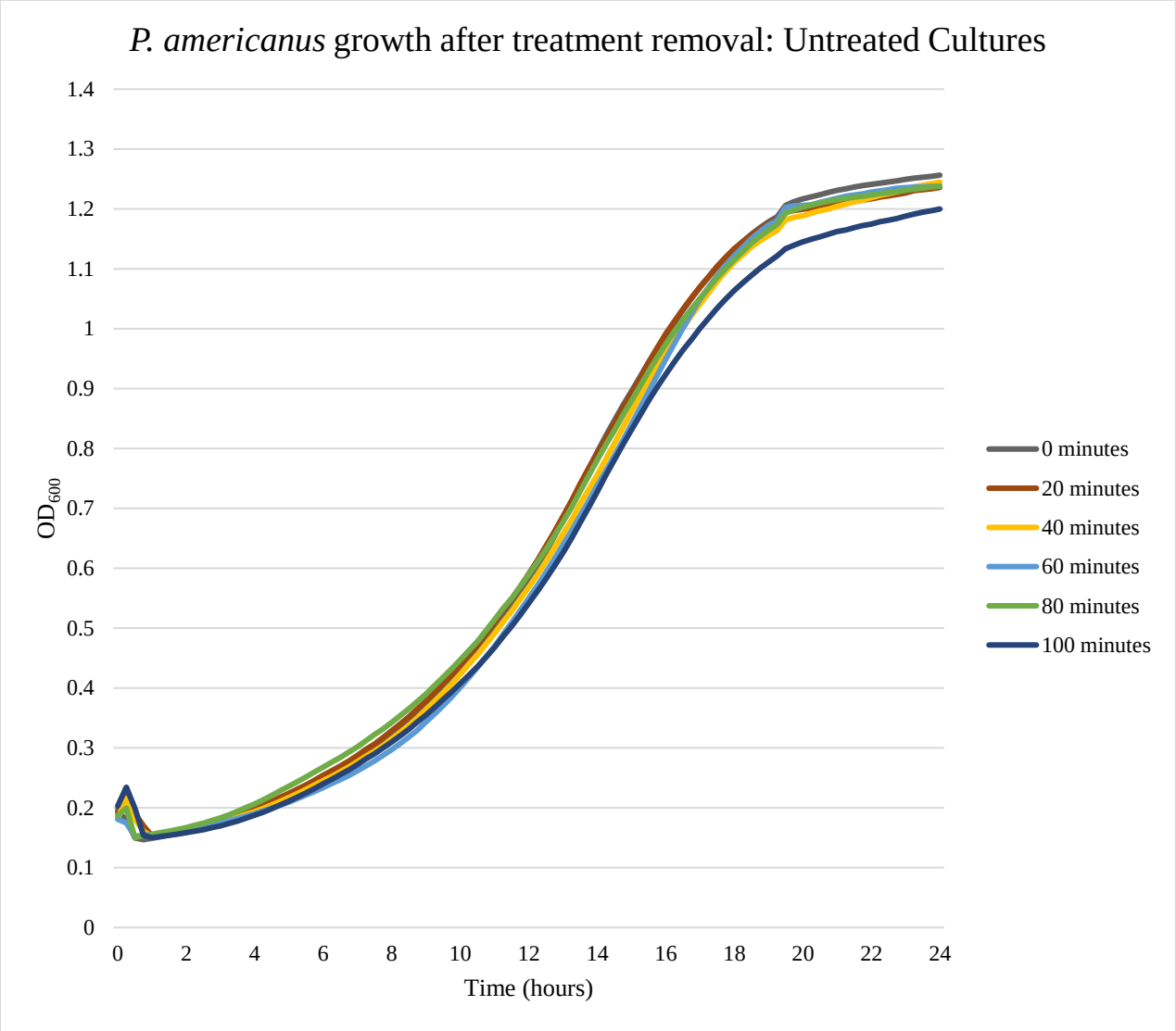

Figure 10: Recovery growth curve of *P. americanus* cultures after exposure to 0.9% saline. Saline was removed via centrifugation prior to growth curve analysis. Each curve indicates the average of three replicates from three experiments for a total of nine replicates up to 19 hours and 15 minutes; afterwards, the curves indicate the average of three replicates from two experiments for a total of six replicates (see Table 3).

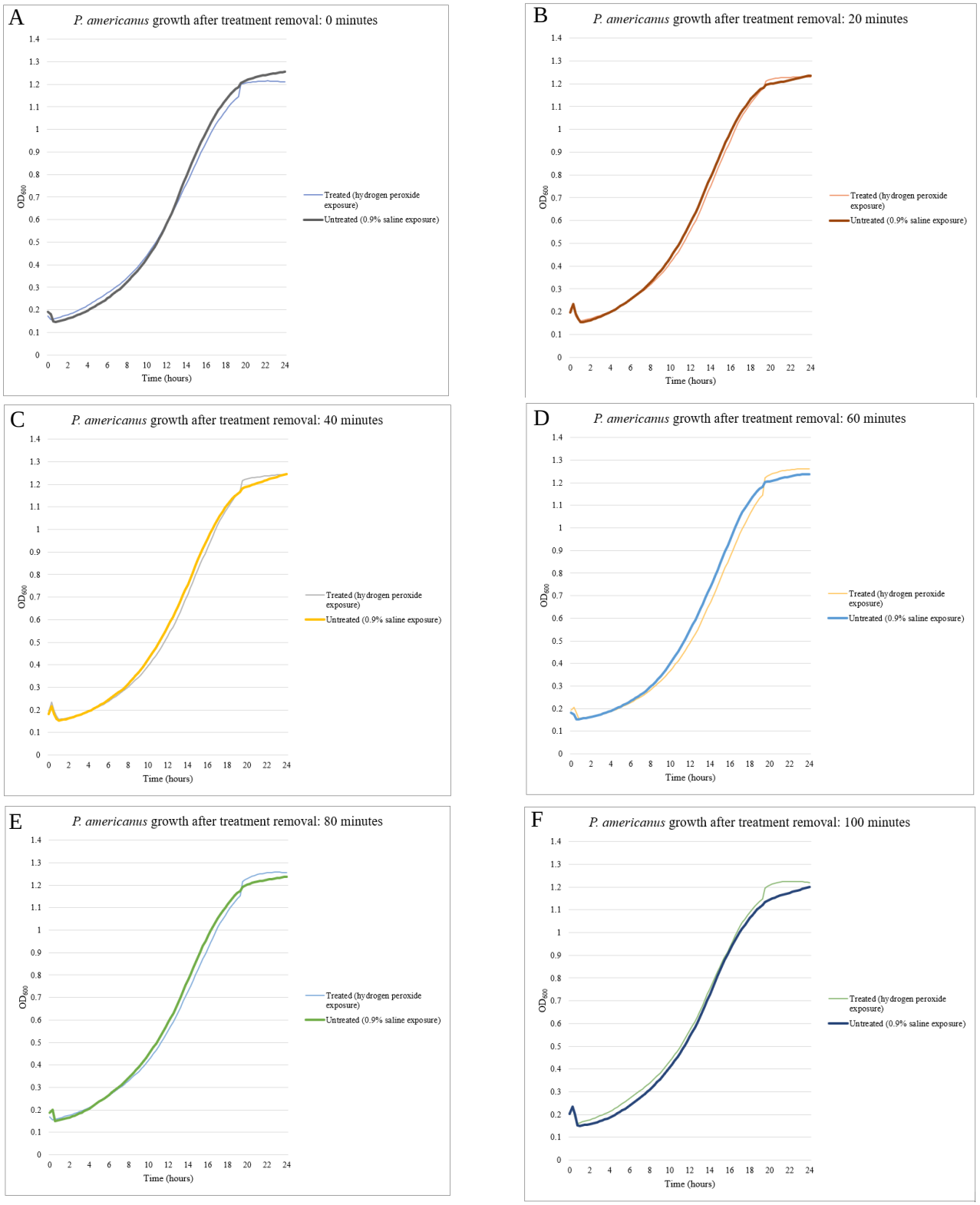

Figure 11: Recovery growth curve of *P. americanus* cultures after (A) 0, (B) 20, (C) 40, (D) 60, (E) 80, and (F) 100 minutes of exposure to H_2O_2 (Treated) or 0.9% saline (Untreated). H_2O_2 or 0.9% saline was removed via centrifugation prior to growth curve analysis. Each curve indicates the average of three replicates of three experiments for a total of nine replicates up to 19 hours and 15 minutes; afterwards, the curves indicate the average of three replicates of two experiments for a total of six replicates (see Table 3).

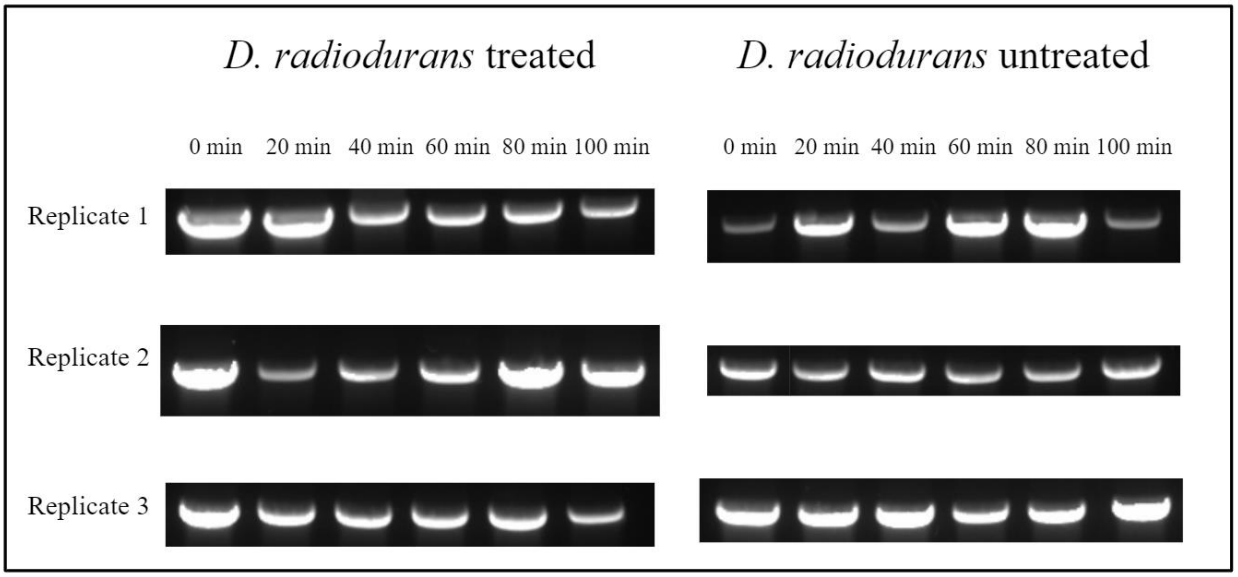

Figure 12: Agarose gel of *D. radiodurans* PCR products. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. See Table 3 for the timing of each replicate. See Figures 14-17 for original gel images.

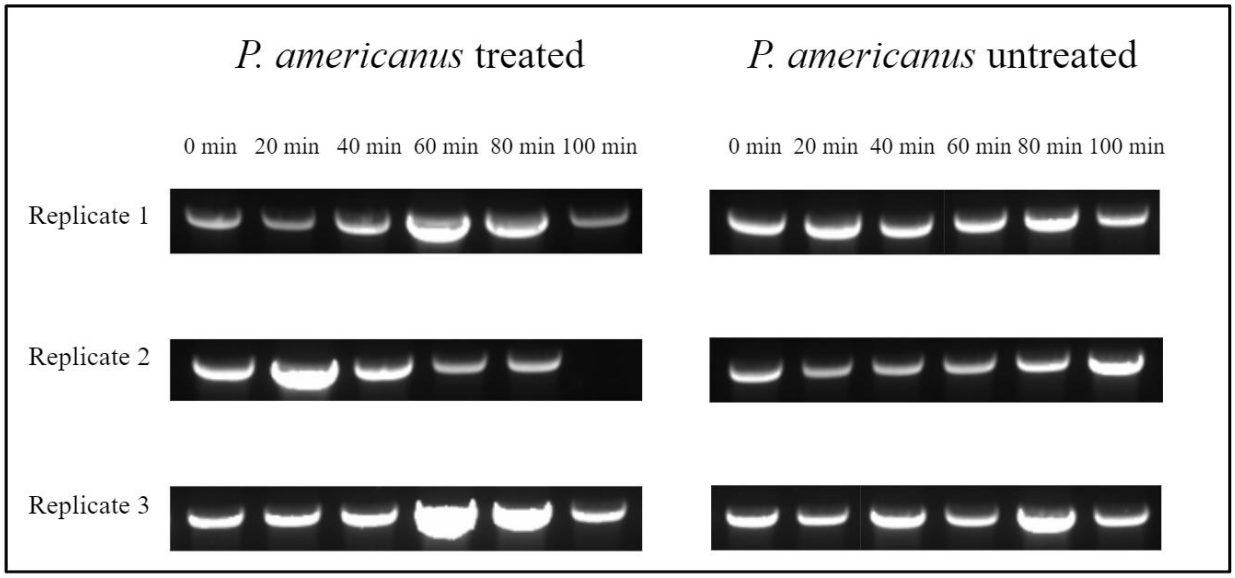

Figure 13: Agarose gel of *P. americanus* PCR products. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. See Table 3 for the timing of each replicate. See Figures 14-17 for original gel images.

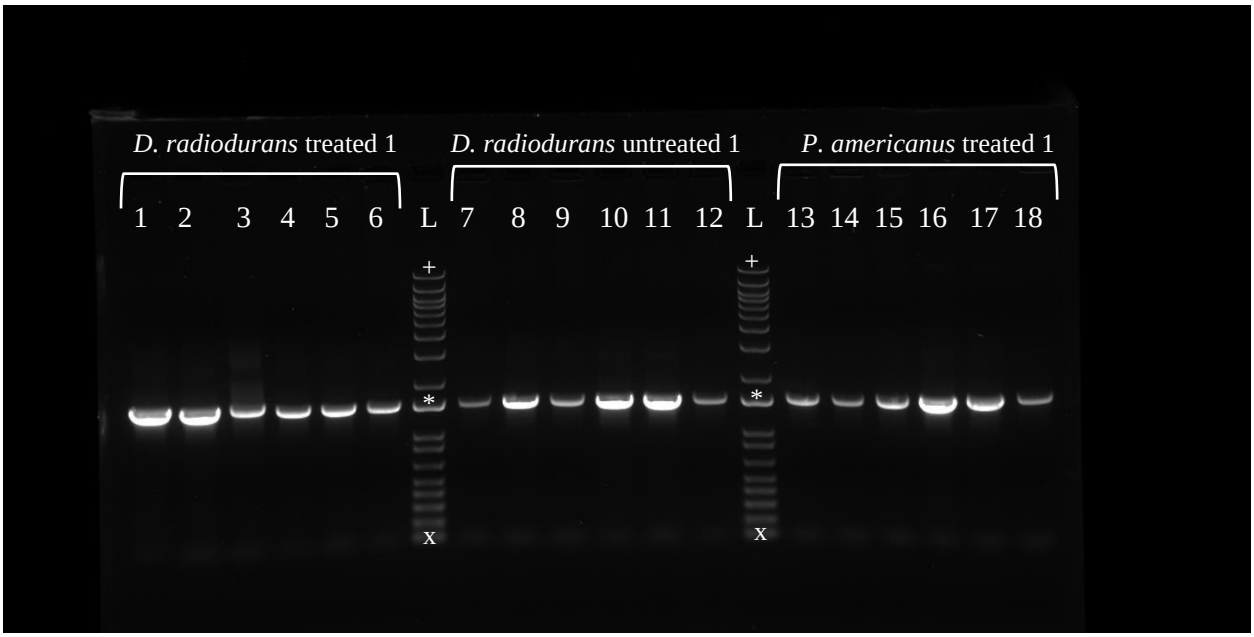

Figure 14: Agarose gel of PCR products. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. Lanes 1-6 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min treated *D. radiodurans* sample. Lanes 7-12 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min untreated *D. radiodurans* samples. Lanes 13-18 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min treated *P. americanus* samples. L indicates Invitrogen DNA 1 kb Plus Ladder. + indicates 15,000 base pairs, * indicates 1,500 base pairs, and x indicates 100 base pairs. "1" indicates that this was the first of three replicate experiments (see Table 3).

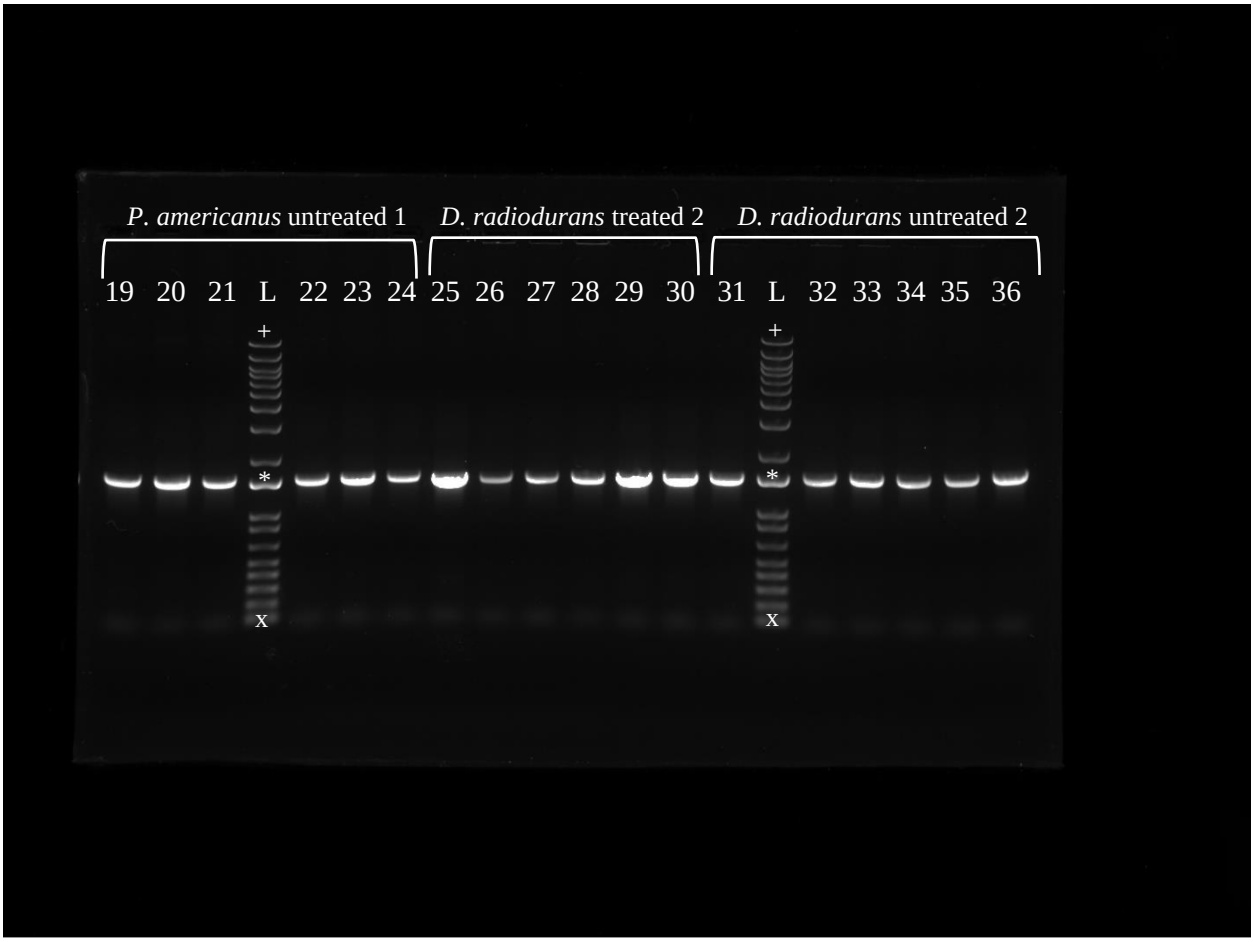

Figure 15: Agarose gel of PCR products. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. Lanes 19-24 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min untreated *P. americanus* sample. Lanes 25-30 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min treated *D. radiodurans* samples. Lanes 31-36 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min untreated *D. radiodurans* samples. L indicates Invitrogen DNA 1 kb Plus Ladder. + indicates 15,000 base pairs, * indicates 1,500 base pairs, and x indicates 100 base pairs. "1" indicates that this was the first of three replicate experiments, and "2" indicates that this was the second of three replicates (see Table 3).

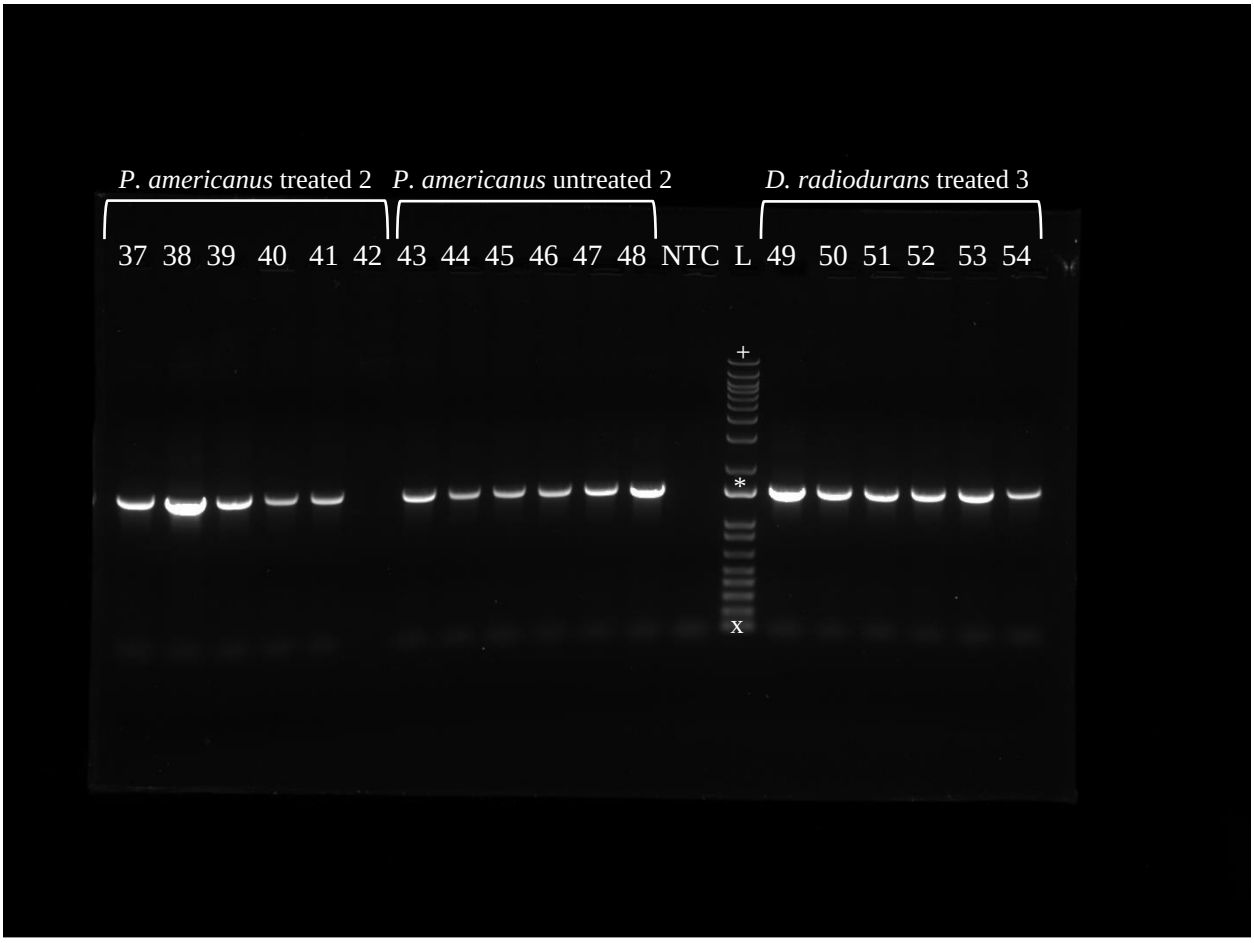

Figure 16: Agarose gel of PCR products. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. Lanes 37-42 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min treated *P. americanus* sample. Lanes 43-48 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min untreated *P. americanus* samples. Lanes 49-54 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min treated *D. radiodurans* samples. L indicates Invitrogen DNA 1 kb Plus Ladder. + indicates 15,000 base pairs, * indicates 1,500 base pairs, and x indicates 100 base pairs. "2" indicates that this was the second of three replicate experiments, and "3" indicates that this was the third of three replicates (see Table 3). NTC indicates a "no template control," in which no DNA template was added.

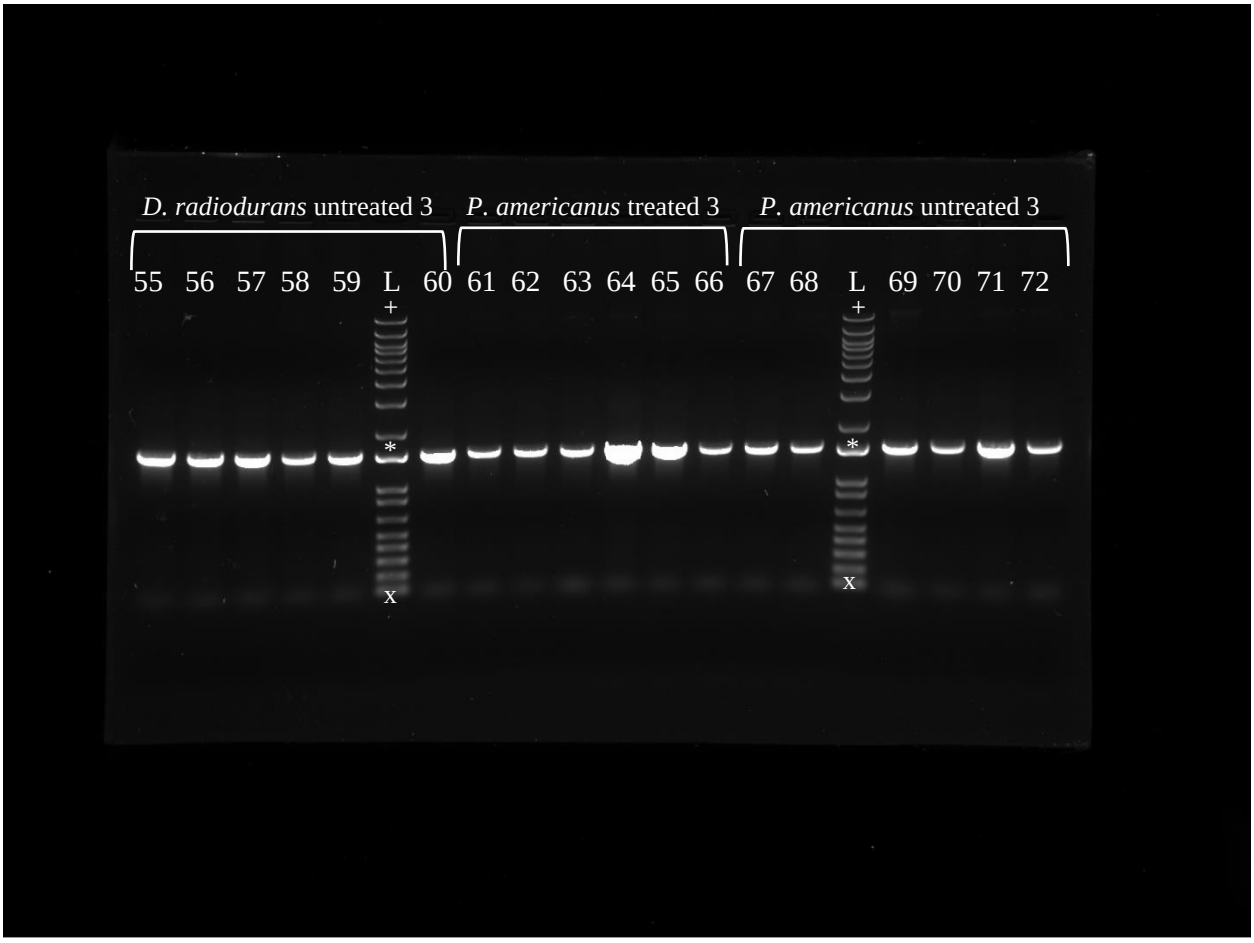

Figure 17: Agarose gel of PCR products. "Treated" indicates exposure to H_2O_2, while "untreated" indicates exposure to 0.9% saline. Lanes 55-60 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min untreated *D. radiodurans* sample. Lanes 61-66 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min treated *P. americanus* samples. Lanes 67-72 indicate (in order) 0-, 20-, 40-, 60-, 80-, and 100-min untreated *P. americanus* samples. L indicates Invitrogen DNA 1 kb Plus Ladder. + indicates 15,000 base pairs, * indicates 1,500 base pairs, and x indicates 100 base pairs. "3" indicates that this was the third of three replicate experiments (see Table 3).

Table 1: Composition of BG11-10x PGY media. 20 g of agar was added to this media for creation of plates.

BG11-10x PGY Media Composition	
Material	**Amount (per L)**
$NaNO_3$	1.5 g
$K_2HPO_4 \cdot 3H_2O$	40 mg
$MgSO_4 \cdot 7H_2O$	75 mg
$CaCl_2 \cdot 2\ H_2O$	36 mg
Citric Acid	6 mg
Ferric Ammonium Citrate	6 mg
Disodium magnesium EDTA	1 mg
Na_2CO_3	20 mg
H_3BO_3	2.86 mg
$MnCl_2 \cdot 4\ H_2O$	1.81 mg
$ZnSO_4 \cdot 7\ H_2O$	222 μg
$Na_2MoO_4 \cdot 2\ H_2O$	390 μg
$CuSO_4 \cdot 5\ H_2O$	79 μg
$Co(NO_3)_2 \cdot 6\ H_2O$	49 μg
Peptone	1.5 g
Yeast Extract	1.5 g
Glucose	1.5 g

Table 2: Composition of TGY media. 15 g of Bacto agar was added to this media for the creation of plates.

TGY Media Composition	
Material	**Amount (per L)**
Tryptone	5 g
Glucose	1 g
Yeast Extract	3 g

Table 3: Dates and replicates of each experiment. X indicates that the procedure was completed on that date.

* Growth curves of *P. americanus* on 3/19/21 were stopped after 19 hours and 15 minutes rather than 24 hours in order to measure optical density of the experimental cultures for 3/20/21. Growth curves were resumed afterwards, but the measurements were off by several minutes when compared to growth curves taken on other dates, so optical density measurements after 19 hours and 15 minutes were excluded from analysis. As a result, *P. americanus* growth curves include three replicates up to 19 hours and 15 minutes, and two replicates at all subsequent time points.

** CFUs on plate at the 20, 60, and 80 minute time points could not be counted due to an error in dilutions that resulted in no growth on necessary dilution plates.

*** Cultures of treated *P. americanus* were not plated for survival analysis on 4/4/21 due to a lack of available plates.

Date	Experiments Performed	Plated for Survival Analysis	Growth Curve Analysis
3/17/21	*D. radiodurans* treated	X	X
	D. radiodurans untreated	X	X
3/19/21	*D. radiodurans* treated	X	X
	D. radiodurans untreated	X	X
	P. americanus treated	X	X*
	P. americanus untreated	X	X*
3/20/21	*D. radiodurans* treated	X	X
	D. radiodurans untreated	X	X
3/21/21	*P. americanus* treated	X	X
	P. americanus untreated	X	X**
4/4/21	*P. americanus* treated***		X
	P. americanus untreated	X	X

Table 4: p-values of comparisons between recovery growth curves of *D. radiodurans* treated and untreated strains at each time point. "Treated" indicates exposure to H_2O_2, and "Untreated" indicates exposure to 0.9% saline. Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (see Materials and Methods). 100 permutations were used for the analysis. Three replicates of three experiments (a total of nine growth curves per exposure group and time point) were analyzed by the software. * indicates statistical significance.

Group One	Group Two	p-value
Treated: 0 minutes of exposure	Untreated: 0 minutes of exposure	0.01
Treated: 20 minutes of exposure	Untreated: 20 minutes of exposure	0.02
Treated: 40 minutes of exposure	Untreated: 40 minutes of exposure	<0.01*
Treated: 60 minutes of exposure	Untreated: 60 minutes of exposure	<0.01*
Treated: 80 minutes of exposure	Untreated: 80 minutes of exposure	<0.01*
Treated: 100 minutes of exposure	Untreated: 100 minutes of exposure	<0.01*

Table 5: p-values of comparisons between recovery growth curves of *D. radiodurans* treated strains at each time point. "Treated" indicates exposure to H_2O_2. Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (see Materials and Methods). 100 permutations were used for the analysis. Three replicates of three experiments (a total of nine growth curves per exposure group and time point) were analyzed by the software.

Group One	Group Two	p-value
Treated: 0 minutes of exposure	Treated: 20 minutes of exposure	0.83
Treated: 0 minutes of exposure	Treated: 40 minutes of exposure	0.50
Treated: 0 minutes of exposure	Treated: 60 minutes of exposure	0.13
Treated: 0 minutes of exposure	Treated: 80 minutes of exposure	0.06
Treated: 0 minutes of exposure	Treated: 100 minutes of exposure	0.32
Treated: 20 minutes of exposure	Treated: 40 minutes of exposure	0.35
Treated: 20 minutes of exposure	Treated: 60 minutes of exposure	0.11
Treated: 20 minutes of exposure	Treated: 80 minutes of exposure	0.11
Treated: 20 minutes of exposure	Treated: 100 minutes of exposure	0.22
Treated: 40 minutes of exposure	Treated: 60 minutes of exposure	0.28
Treated: 40 minutes of exposure	Treated: 80 minutes of exposure	0.17
Treated: 40 minutes of exposure	Treated: 100 minutes of exposure	0.89
Treated: 60 minutes of exposure	Treated: 80 minutes of exposure	0.97
Treated: 60 minutes of exposure	Treated: 100 minutes of exposure	0.21
Treated: 80 minutes of exposure	Treated: 100 minutes of exposure	0.46

Table 6: p-values of comparisons between recovery growth curves of *D. radiodurans* untreated strains at each time point. "Untreated" indicates exposure to 0.9% saline. Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (see Materials and Methods). 100 permutations were used for the analysis. Three replicates of three experiments (a total of nine growth curves per exposure group and time point) were analyzed by the software.

Group One	Group Two	p-value
Untreated: 0 minutes of exposure	Untreated: 20 minutes of exposure	0..72
Untreated: 0 minutes of exposure	Untreated: 40 minutes of exposure	0..43
Untreated: 0 minutes of exposure	Untreated: 60 minutes of exposure	0..41
Untreated: 0 minutes of exposure	Untreated: 80 minutes of exposure	0.28
Untreated: 0 minutes of exposure	Untreated: 100 minutes of exposure	0.14
Untreated: 20 minutes of exposure	Untreated: 40 minutes of exposure	0.68
Untreated: 20 minutes of exposure	Untreated: 60 minutes of exposure	0.77
Untreated: 20 minutes of exposure	Untreated: 80 minutes of exposure	0.30
Untreated: 20 minutes of exposure	Untreated: 100 minutes of exposure	0.25
Untreated: 40 minutes of exposure	Untreated: 60 minutes of exposure	0.48
Untreated: 40 minutes of exposure	Untreated: 80 minutes of exposure	0.73
Untreated: 40 minutes of exposure	Untreated: 100 minutes of exposure	0.55
Untreated: 60 minutes of exposure	Untreated: 80 minutes of exposure	0.07
Untreated: 60 minutes of exposure	Untreated: 100 minutes of exposure	0.19
Untreated: 80 minutes of exposure	Untreated: 100 minutes of exposure	0.53

Table 7: p-values of comparisons between recovery growth curves of *P. americanus* treated and untreated strains at each time point. "Treated" indicates exposure to H_2O_2, and "Untreated" indicates exposure to 0.9% saline. Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (see Materials and Methods). 100 permutations were used for the analysis. Three replicates of three experiments (a total of nine growth curves per exposure group and time point) were analyzed by the software up to 19 hours and 15 minutes; afterwards, three replicates of two experiments (a total of six growth curves per exposure group and time point) were compared (see Table 3).

Group One	Group Two	p-value
Treated: 0 minutes of exposure	Untreated: 0 minutes of exposure	0.77
Treated: 20 minutes of exposure	Untreated: 20 minutes of exposure	0.75
Treated: 40 minutes of exposure	Untreated: 40 minutes of exposure	0.86
Treated: 60 minutes of exposure	Untreated: 60 minutes of exposure	0.66
Treated: 80 minutes of exposure	Untreated: 80 minutes of exposure	0.75
Treated: 100 minutes of exposure	Untreated: 100 minutes of exposure	0.24

Table 8: p-values of comparisons between recovery growth curves of *P. americanus* treated strains at each time point. "Treated" indicates exposure to H_2O_2. Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (see Materials and Methods). 100 permutations were used for the analysis. Three replicates of three experiments (a total of nine growth curves per exposure group and time point) were analyzed by the software up to 19 hours and 15 minutes; afterwards, three replicates of two experiments (a total of six growth curves per exposure group and time point) were compared (see Table 3).

Group One	Group Two	p-value
Treated: 0 minutes of exposure	Treated: 20 minutes of exposure	0.77
Treated: 0 minutes of exposure	Treated: 40 minutes of exposure	0.48
Treated: 0 minutes of exposure	Treated: 60 minutes of exposure	0.30
Treated: 0 minutes of exposure	Treated: 80 minutes of exposure	0.98
Treated: 0 minutes of exposure	Treated: 100 minutes of exposure	0.97
Treated: 20 minutes of exposure	Treated: 40 minutes of exposure	0.59
Treated: 20 minutes of exposure	Treated: 60 minutes of exposure	0.27
Treated: 20 minutes of exposure	Treated: 80 minutes of exposure	0.97
Treated: 20 minutes of exposure	Treated: 100 minutes of exposure	0.93
Treated: 40 minutes of exposure	Treated: 60 minutes of exposure	0.69
Treated: 40 minutes of exposure	Treated: 80 minutes of exposure	0.58
Treated: 40 minutes of exposure	Treated: 100 minutes of exposure	0.26
Treated: 60 minutes of exposure	Treated: 80 minutes of exposure	0.30
Treated: 60 minutes of exposure	Treated: 100 minutes of exposure	0.20
Treated: 80 minutes of exposure	Treated: 100 minutes of exposure	0.75

Table 9: p-values of comparisons between recovery growth curves of *P. americanus* untreated strains at each time point. "Untreated" indicates exposure to 0.9% saline. Growth curves were analyzed using the CGGC (Compare Groups of Growth Curves) permutation test tool designed by Gordon Smyth, Russell Thompson and Keith Satterley (see Materials and Methods). 100 permutations were used for the analysis. Three replicates of three experiments (a total of nine growth curves per exposure group and time point) were analyzed by the software up to 19 hours and 15 minutes; afterwards, three replicates of two experiments (a total of six growth curves per exposure group and time point) were compared (see Table 3).

Group One	Group Two	p-value
Untreated: 0 minutes of exposure	Untreated: 20 minutes of exposure	0.85
Untreated: 0 minutes of exposure	Untreated: 40 minutes of exposure	0.63
Untreated: 0 minutes of exposure	Untreated: 60 minutes of exposure	0.30
Untreated: 0 minutes of exposure	Untreated: 80 minutes of exposure	0.80
Untreated: 0 minutes of exposure	Untreated: 100 minutes of exposure	0.23
Untreated: 20 minutes of exposure	Untreated: 40 minutes of exposure	0.50
Untreated: 20 minutes of exposure	Untreated: 60 minutes of exposure	0.32
Untreated: 20 minutes of exposure	Untreated: 80 minutes of exposure	1.00
Untreated: 20 minutes of exposure	Untreated: 100 minutes of exposure	0.18
Untreated: 40 minutes of exposure	Untreated: 60 minutes of exposure	0.73
Untreated: 40 minutes of exposure	Untreated: 80 minutes of exposure	0.57
Untreated: 40 minutes of exposure	Untreated: 100 minutes of exposure	0.46
Untreated: 60 minutes of exposure	Untreated: 80 minutes of exposure	0.30
Untreated: 60 minutes of exposure	Untreated: 100 minutes of exposure	0.56
Untreated: 80 minutes of exposure	Untreated: 100 minutes of exposure	0.50