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The impact of desiccation and freezing on gamma radiation survival in microorganisms

by

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Thesis submitted to the Faculty of the
Molecular and Cell Biology Graduate Program,
Uniformed Services University of the Health Sciences
in partial fulfillment of the requirements for the degree of
Master of Science 2020



March 19, 2020

APPROVAL SHEET

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Date 03/19/2020

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ACKNOWLEDGEMENTS

I'd like to start by thanking Drs. Elena Gaidamakova and Vera Matrosova for sharing their years of expertise with me and holding me to a high standard throughout this study. I also want to thank Dr. Rok Tkavc for his instruction on proper microorganism lab technique and the time he took to make sure I mastered these skills. And I would be remiss in not mentioning Robert Volpe, whose creative thinking and willingness to explore new concepts sparked that same fire in me.

As for collaborators and outside assistance, I want to recognize the efforts of Dr. Peter Setlow and research associate George Korza of the University of Connecticut School of Health. Their assistance in culturing, harvesting, and counting *Bacillus* spore strains helped generate invaluable data for this study. I also wish to thank Dr. Cara Olsen and Sorana Raiciulescu of the Uniformed Services University (USU) Biostatistics Consulting Center for their guidance and recommendations for conducting a thorough statistical analysis of my data. Additional recognition goes to Dr. J. Dumler and the entire USU Department of Pathology for the shared resources, expertise, and encouragement throughout this study; as well as Michael Woolbert and the staff of the Radiation Safety Division of the USU Environmental Health and Safety office for maintain the irradiators that were vital to this study.

I want to thank the U.S. Department of Defense for giving me the opportunity to study at this outstanding university. I especially thank the Defense Threat Reduction Agency for their financial support of this research through grant HU001-18-2-0034.

Special thanks go to my mentor and primary investigator, Dr. Michael Daly. His vast knowledge of radiation microbiology, coupled with his boundless motivation and incredible work ethic, drove me to give my best throughout the study and expand my understanding of the mechanisms of ionizing radiation effects on organisms. I promise, if I find any other Soldiers like me, I'll send them your way!

Finally, I want to say thank you to the most important people in my life: my children and my wife, Darcie. Through numerous long lab days, nights filled with homework and papers, and all the stress and anxiety these events bring, you supported me, encouraged me, and helped me achieve a level of greatness I didn't know I could reach. I love you all, and I look forward to sharing our next great adventure together.

DEDICATION

To my children, Coulson, Anthony, and Penelope.

To my mother and father, and to my grandmother, Jane.

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ABSTRACT

The impact of desiccation and freezing on gamma radiation survival in microorganisms

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Gamma radiation and desiccation cause oxidative stress in microorganisms, mediated by reactive oxygen species (ROS) generated by abiotic and metabolic mechanisms. During irradiation, ROS are produced in cells by water radiolysis; during desiccation, by exposure to atmospheric oxygen and dysfunctional metabolic processes. Bacteria and yeasts that are radiation-resistant are typically also desiccation-resistant and share a great antioxidant capacity. This thesis considers how different physicochemical environments that affect cellular water content and state influence radiation resistance. The gamma radiation survival metrics of eight model microorganisms (three vegetative bacterial strains (*Escherichia coli*, *Acinetobacter baumannii*, *Lactobacillus plantarum*), three bacterial spore strains (*Bacillus megaterium*, *Bacillus thuringiensis*, *Bacillus subtilis*), and two budding yeast strains (*Saccharomyces cerevisiae* EXF-6761 and *S. cerevisiae* EXF-6218)) are reported for the following physicochemical states: as liquid cultures irradiated on wet ice (0°C); as frozen-aqueous cultures irradiated on dry ice (-79°C); as desiccated cells at room temperature irradiated on wet ice; and as frozen-desiccated cells irradiated on dry ice. The survival data show that desiccating or freezing

most vegetative cells before irradiation extends their resistance to gamma radiation. In contrast, *Bacillus* spores with naturally low intracellular water contents did not display increased radiation resistance upon desiccation or freezing; and *L. plantarum*, which lacks antioxidant enzymes, only displayed increases in radiation resistance upon freezing. The phenomenon of desiccation- and freezing-facilitated radiation resistance is important to the applied scientific disciplines of radiation sterilization by promoting new protocols to optimize sterilization yields, biodefense by demonstrating the dangers frozen and desiccated biological agent preparations pose to current protection measures, and space exploration by understanding how environmental conditions in space could facilitate microorganism survival against cosmic radiation and unintended transportation between worlds.

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CHAPTER 1: Introduction

IONIZING RADIATION

Ionizing radiation and its place in our lives.

Ionizing radiation is a type of energy generated by either the nuclear decay of unstable elements, called radionuclides, or the impact of free electrons on the outer electron shell of an atom. This energy travels either as particles (alpha, beta, neutrons) or electromagnetic waves (gamma, X-rays) and is strong enough to strip electrons from other atoms or molecules, thereby ionizing them. Ionizing radiation, by its nature, can be dangerous or lethal to living organisms at high enough doses. However, when properly regulated and used in a careful and controlled manner, radiation has many beneficial applications, including sterilization, medical treatment and imaging, and energy production [1, 2].

Sterilization. For decades, gamma radiation has been used to sterilize food and medical supplies in multiple nations and continents. The International Atomic Energy Agency (IAEA) reported in 2008 that over 200 gamma irradiators were in use worldwide to irradiate a variety of products [3]. By 2010, almost 400,000 tons of food products were irradiated annually in Asia, the European Union, and the United States combined [4]. Currently, approximately 12,000,000 m³ of medical devices, including more than 40% of all single-use medical devices worldwide, are sterilized by ionizing radiation annually [5]. Although recent research indicates the possibility of deleterious long-term

effects of utilizing some irradiated products [6], ionizing radiation continues to be a commonly-used option for sterilization.

Medical imaging and treatment. Medical applications of ionizing radiation are the highest man-made contributor to average annual radiation exposure across the globe, with people in the US receiving approximately 3.1 millisieverts (mSv) of radiation, equal to 3.1 milligray (mGy) of gamma radiation, per year from imaging and radiation therapy [7, 8]. As of 2007, over 3.6 billion medical imaging procedures are performed annually worldwide [9], and a 2019 study of health insurance billing documents from hospitals in the US and Canada showed there were about 160-163 radiation-based imaging tests performed for every 1,000 patients seen in 2016 [10]. Looking at rates in the US, approximately 24% of all cancer patients received radiation therapy in the year 2000, and current recommendations indicate as many as half of all cancer patients could benefit from radiation therapy of some form [11].

Energy production. In 1943, the US Government constructed the world's first electricity-producing nuclear reactor in what is now the Oak Ridge National Laboratory (ORNL) in Tennessee [12]. Since then, the use of nuclear radiation to produce energy has greatly expanded. Today, there are approximately 440 operating nuclear reactors around the world, providing 10% of the world's electricity, as well as radionuclides for the industrial and medical purposes previously described [13]. Safety remains a concern regarding these facilities, however, as major accidents at Three Mile Island, USA in 1979 [14]; Chernobyl, Ukraine in 1986 [15]; and Fukushima, Japan in 2011 [16] demonstrate.

Environmental sources. On average, Americans are exposed to approximately 3.1 mSv of radiation from environmental sources per year [7]. Terrestrial sources of

environmental radiation exposure include inhaled radon gas, radionuclides in the earth (potassium-40, uranium-238, thorium-232, and their radioactive products), and radionuclides in our bodies (primarily potassium-40) [17]. Cosmic radiation is the only non-terrestrial source of background ionizing radiation, consisting of protons (89%), alpha particles (10%), and nuclei from heavier elements (1%); however, much of this energy is deflected by Earth's magnetosphere or absorbed in the upper layers of the atmosphere [18].

The ever-changing face of ionizing radiation damage.

With its level of importance in everyday life, it is critically important that we understand the intricate nature of how ionizing radiation interacts with living organisms. However, since the turn of the 20th century, when the works of Röntgen, Becquerel, the Curies, and Rutherford introduced the world to the concepts of radioactivity and ionizing radiation [19], our understanding of these interactions has changed significantly.

Direct and indirect effects. In his seminal 1987 review “The Chemical Basis of Radiation Biology” [20], von Sonntag noted two different mechanisms by which ionizing radiation damaged organic molecules. The first mechanism, dubbed the “direct effect”, involved radiation impacting the electrons of the molecule directly, ejecting the electron from the molecule and turning it into an ionized radical with altered structure and reactivity. In the second mechanism, or “indirect effect”, radiation impacts an electron in a water molecule, ejecting the electron and forming a hydrogen ion and hydroxyl radical in a process called radiolysis. The soluble electron and hydroxyl radical can either react directly with an organic molecule to oxidize it or react with another hydroxyl radical to

form hydrogen peroxide (H_2O_2). Hydrogen peroxide in cells is readily converted to O_2 through heavy metal catalyzed reactions, yielding superoxide ($\text{O}_2^{\cdot-}$) indirectly from ionizing radiation. Together, hydroxyl radicals, superoxide, and hydrogen peroxide form a class of molecules called reactive oxygen species (ROS) [20-22].

How does ionizing radiation cause injury? The concept of radiolysis had been studied for over a century [23]; however, the relative importance of direct versus indirect effects on radiation biology and survival was a topic of debate even in the 1980s [20]. This debate was related to another point of contention in radiation biology: what molecule or molecules in the cell are the most important target of ionizing radiation? In 1966, Hutchinson argued that DNA was the crucial target of ionizing radiation, with its damage being the basis for the biological changes and death noted from radiation exposure [24]. Other notable scientists in the field agreed with Hutchinson [20, 25, 26], and much of radiation biology at the time focused on DNA damage [27-34].

DNA damage from ionizing radiation can be either a base pair modification, a single-strand break (SSB), or a double-strand break (DSB) [25, 26]; these lesions can be further classified as complex (base pair modification next to SSB or DSB) or clustered (multiple non-DSB lesions located within one or two DNA helix turns) [33]. Of these, simple and complex DSBs and clustered lesions were seen as the most predictive of cell death from ionizing radiation [33, 34]. At high enough levels, these lesions result in disruptions to metabolic pathways, cell membranes and organelles, and replication, leading to cell death [35]. Lower numbers of lesions, repaired through non-homologous end joining (NHEJ), can alter a cell's genome, resulting in possible mutations [36], chromosomal aberrations [33, 37], and cancerous growth [38, 39]. Eukaryotic cells also

have unique pathways that detect DSB damage to trigger either cellular senescence or apoptosis. Marker proteins such as ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) are recruited to DSB sites [40, 41] and phosphorylate p53, which induces either senescence or apoptosis depending on the level of signaling [42-45].

So, which effect, direct or indirect, is the primary source for these DNA lesions? Research indicates that greater than 80% of the ionizing radiation energy entering a cell is absorbed by intracellular water [46]. However, near the DNA strand itself, water absorbs only about 60% of the incoming energy [25]. Studies also show that hydroxyl radicals account for most, but not all, of the SSBs and DSBs noted in aqueous irradiated DNA preparations [27, 34]. It has been shown that electrons, released either from the radiolysis of water [30, 31] or the ionization of atoms in the DNA strand itself [28, 29], can also generate complex SSB and DSB lesions. These findings suggest that both direct and indirect methods of ionizing radiation damage are involved in DNA damage. Indeed, even von Sonntag posited that "... radiation-induced damage of DNA will result from the direct effect but that a contribution by the solvation shell cannot be neglected [20]."

Is it just DNA? Although DNA damage does correlate with cell death from ionizing radiation, it does not do so in a predictable manner across organisms. For example, many bacterial species are killed by low doses of gamma radiation that generate few, if any, DSBs, but other species can survive high doses that generate hundreds of DSBs [47]. Similarly in mammalian cells, DNA damage assays are unreliable in predicting the radiosensitivity of tumor cells [48]. In fact, rates of DNA damage between organisms of varying radiosensitivities are very similar when normalized to genome size

and radiation dose (DSB/Gy/Mbp) [49]. In order to identify additional factors that could influence DNA damage toxicity in cells, researchers studied radiation-resistant organisms, such as *Deinococcus radiodurans* and hyperthermophilic archaea, to identify what additional genomic or proteomic characteristics these organisms share that could result in their radioresistant phenotype [50-53]. Numerous focused studies of ionizing radiation repair mechanisms in *D. radiodurans* reported the critical role of RecA-dependent and RecA-independent recombination pathways in recovery [52, 54, 55]. Alignment of the multiple chromosomes in *D. radiodurans* could also account for improved DSB repair after radiation exposure [52, 56]. However, sequencing the *D. radiodurans* genome revealed it to be very similar to those of other, less radiation-resistant, organisms, with no significant differences in the assortment of DNA repair genes to account for the pronounced differences in resistance [51]. Indeed, *D. radiodurans* mutants with a deficient DNA polymerase gene that rendered the organism radiation-sensitive had their resistance restored with the introduction of a homologous gene from *Escherichia coli*, an organism with much lower radiation resistance [50].

Further study into *D. radiodurans* revealed the organism maintained an unusually high ratio of manganous (II) (Mn(II)) ions versus iron (II) (Fe(II)) ions (Mn/Fe). By contrast, radiation-sensitive organisms, such as *Shewanella oneidensis*, had very low Mn/Fe ratios [47, 57]. Fe(II) has long been known to facilitate hydroxyl radical formation from hydrogen peroxide in what is named the “Fenton reaction” [20], making it a potent agent for inducing oxidative damage in cells. More recent studies demonstrated that Mn(II) supplementation alone has an antioxidant effect in cells, specifically in protecting proteins from oxidative modification by carbonyl groups

(carbonylation) initiated by superoxide (**Fig. 1**) [58]. The act of protein protection correlated surprisingly well to radiation resistance in multiple studies [47, 58-60]. Indeed, protein damage from other sources [61] and repair proteins rendered defective by mutation [39] are highly predictive of radiation sensitivity. Together, these studies demonstrated that proteins are as important a target of ionizing radiation as DNA, if not more so, in regards to ionizing radiation resistance and survival [22, 49]. Because protein damage from irradiation is primarily superoxide-induced carbonylation [59, 61], this underscores the theory that indirect radiation damage through water radiolysis has a far greater role in cell death than direct radiation damage [22].

Ionizing radiation protection.

Evolution of ionizing radiation resistance. Survival to a given dose of ionizing radiation varies dramatically among organisms. Approximately half of all humans will die in 30 days ($LD_{50/30}$) when exposed to 4-5 Sv (4-5 Gy of gamma radiation) [62]; for mice, that dose is 7-8 Gy [63]. Fruit fly (*Drosophila melanogaster*) populations decrease by half, two days after exposure to 1300 Gy ($LD_{50/2}$) [64]; and for the tardigrade *Milnesium tardigradum*, that decrease occurs at 5,000 Gy [65]. Among bacteria, 10% of *E. coli* survive 700 Gy of gamma radiation; in stark contrast, *D. radiodurans* needs a dose of 12 kGy (12,000 Gy) for the same population loss [47].

One surprising fact about higher radiation resistance levels in some organisms is that there is no recognized source of radiation over geologic timespans that can account for their evolution. For example, environmental radiation levels in the U.S. are about 700-800 times less than the $LD_{50/30}$ dose for humans [7, 62], and the outdoor dose rate of

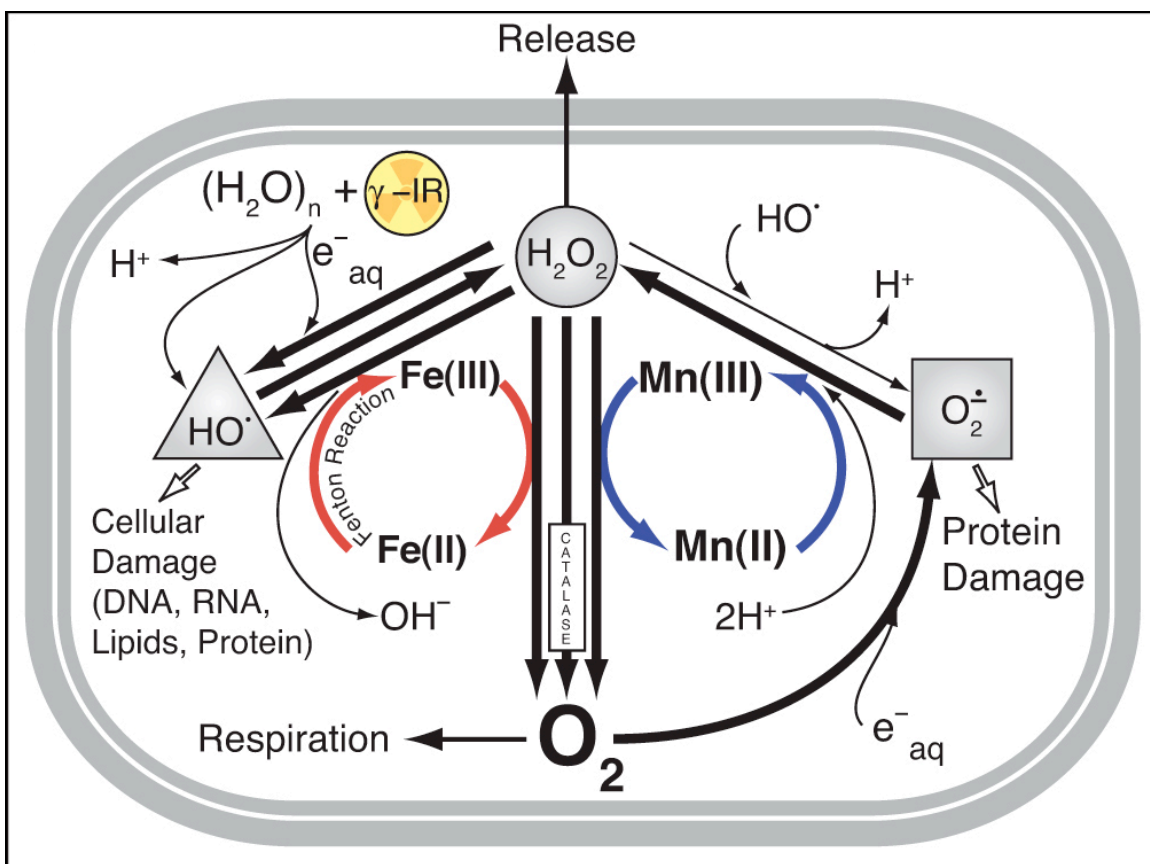


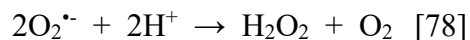
Figure 1. Ionizing radiation-induced ROS formation pathways in the cell. Used with permission from Dr. Michael J. Daly [22].

one of the highest background radiation site in the world (Sichuan, China) is only about 80 mGy/hr [17]. As previously discussed, ionizing radiation's most significant method of injury is through the production of ROS. However, ROS are more commonly encountered as signaling molecules in low concentrations [66, 67], immune responses to pathogens and injury [68], and byproducts of metabolic pathways [35]. Environments with high concentrations of certain heavy metals, such as iron, can also catalyze ROS formation [69].

The most common source of cellular injury that is comparable to ionizing radiation, though, is desiccation [70, 71]. For instance, samples of *D. radiodurans* dehydrated for 6 weeks had the same number of DSBs per genome as samples exposed to

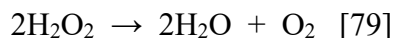
6 kGy of gamma radiation. Like ionizing radiation injury, the cell's ability to repair DNA damage after desiccation is key to the cell's ability to survive desiccation [72, 73]. This is representative of the strong correlation researchers have made between ionizing radiation and desiccation resistance, starting with *D. radiodurans* [71, 73]. Indeed, two studies that analyzed *D. radiodurans* gene induction from either ionizing radiation or desiccation injury demonstrated strong overlap in the hierarchy of expressed genes [74, 75]. Further studies demonstrated that much of the cellular damage caused by desiccation can be traced back to ROS formation, either from disruption of photosynthetic or metabolic electron transport chains or elimination of extracellular hydration shells that prevent atmospheric oxygen entry into the cell [70, 72, 76]. This body of evidence implicates that the evolution of desiccation resistance resulted in the development of ionizing radiation resistance as an unintended side effect.

Antioxidant enzymes. In response to the daily bombardment from ROS, organisms have evolved multiple mechanisms to mitigate or eliminate these chemical species. Two prominent classes of enzymes that evolved to control ROS are superoxide dismutase (SOD) and catalase [35]. SOD is a metalloprotein that contains a heavy metal catalytic ion, either copper, zinc, manganese, or nickel depending on the organism and organelle it is found in [35, 77]. SOD works by catalyzing the dismutation of superoxide radicals into oxygen and hydrogen peroxide:



Versions of SOD are ubiquitous throughout most of the natural world and are used not just to protect cells from oxidative damage, but also modulate several biological processes and signaling pathways [35, 77, 78]. Catalase is another metalloprotein

enzyme found in many organisms [35]. It continues the reduction process started by SOD by converting hydrogen peroxide to water and oxygen:



Nonenzymatic antioxidants. In addition to enzymes, which remove longer-lived ROS species (H_2O_2 , $\text{O}_2^{\bullet-}$), non-enzymatic antioxidants are also present in many organisms to scavenge similar ROS species [80]. These molecules, which include small metabolites (peptides, carboxylic acids, and nucleosides), vitamin C (ascorbate), vitamin E and other tocopherols, β -carotene and other carotenoids, glutathione, flavonoids, and alkaloids, react quickly with different ROS species to prevent their proliferation throughout the cell [35, 81]. Research into radioresistant fungi [82] and certain species of cyanobacteria [83] also revealed possible antioxidant properties in melanin and trehalose.

Manganese complexes (Mn antioxidants). As mentioned earlier, high intracellular levels of manganese, in the form of Mn(II), are indicative of ionizing radiation resistance [22, 47, 80] (**Fig. 1**). While some SOD and catalase variants contain Mn(II) ions, researchers found that protein-free cell isolates from radiation-resistant prokaryotes *D. radiodurans*, *Halobacterium salinarum*, and *Lactobacillus plantarum* that contained high levels of Mn(II) still provided substantial levels of radioprotection to living cells [84, 85]. Further analysis revealed these Mn(II) ions spontaneously formed antioxidant complexes with phosphates, peptides, nucleosides, and other organic metabolites, with the high concentration of Mn(II) in the organism facilitating complex formation [84, 86]. The accumulation of Mn(II)-metabolite complexes, as measured by electron paramagnetic resonance (EPR), has been shown to be a powerful predictor of ionizing radiation resistance across the tree of life [80].

WATER

SOD, catalase, and Mn(II) antioxidants work to reduce and clear ROS from cells.

As demonstrated, however, the vast majority of ionizing radiation-induced ROS are derived from water radiolysis. This raises the question, what would happen to an organism's radiation resistance if its water was altered or removed?

Ways of altering water form and content.

Freezing. Under standard atmospheric conditions (15°C, pressure 101.325 kilopascals or 1 atm), water is primarily in liquid state. In this state, polar and ionic molecules (including ROS) can diffuse freely through the medium. However, when water begins to freeze, its molecules most often assume a rigid, hexagonal lattice structure due to hydrogen bond formation between molecules [87]. In this structure, one would not expect much movement of dissolved contents through the medium, including ROS. Indeed, proteins in liquid preparations sustain far less damage when frozen versus unfrozen [88]. DNA plasmids irradiated in an aqueous solution also experienced more than 100 times as many strand breaks at 25°C instead of -196°C [32, 49]. The field of food science has studied this phenomenon at length, demonstrating how bacteria-treated food supplies require greater radiation doses for decontamination when frozen than when they are not frozen [89-91].

Drying. As previously discussed, researchers have established a strong correlation between desiccation resistance and ionizing radiation resistance [73]. However, for organisms that do survive desiccation, one would suspect that the reduced

intracellular and extracellular water content would reduce ionizing radiation's ability to generate ROS via radiolysis, greatly limiting indirect damage to cell contents.

Researchers have demonstrated this effect in the lab, as well. In the same plasmid study described earlier [32], irradiated lyophilized (dried under extremely low pressure) DNA solutions had over 1,000 times fewer strand breaks than aqueous samples when irradiated at 25°C. Meanwhile, another food irradiation study demonstrated that freeze-dried meat treated with *Salmonella typhimurium*, then rehydrated to a 10% water/solid weight ratio and irradiated, had much greater bacterial survival than treated freeze-dried meat rehydrated to higher water/solid weight ratios and irradiated at the same dose [92].

Proof of concept: *D. radiodurans* pilot study

In 2002, the *National Academy of Sciences* published a pilot radiation survival curve built by Dr. John Battista (LSU) of *D. radiodurans* that had been air desiccated on microscope slides [93]. This survival curve demonstrated that, when desiccated, *D. radiodurans* could survive radiation doses up to 15 Mrad (150 kGy). However, the veracity of these findings was still in question until 2018, when NASA Planetary Protection Officer Dr. Catherine Conley asked Dr. Michael Daly to validate Battista's findings. Daly's study expanded on Battista's work by desiccating and freezing *D. radiodurans* samples to -80°C, then testing their survival following exposure to gamma radiation. The USU study demonstrated that desiccating *D. radiodurans* before irradiation greatly extends its resistance to gamma rays (Co-60) and protons, from a 10% survival value (D_{10}) of 15 kGy in liquid culture to an astonishing D_{10} of 60 kGy when the cells are desiccated and deeply frozen (**Fig. 2**) [94].

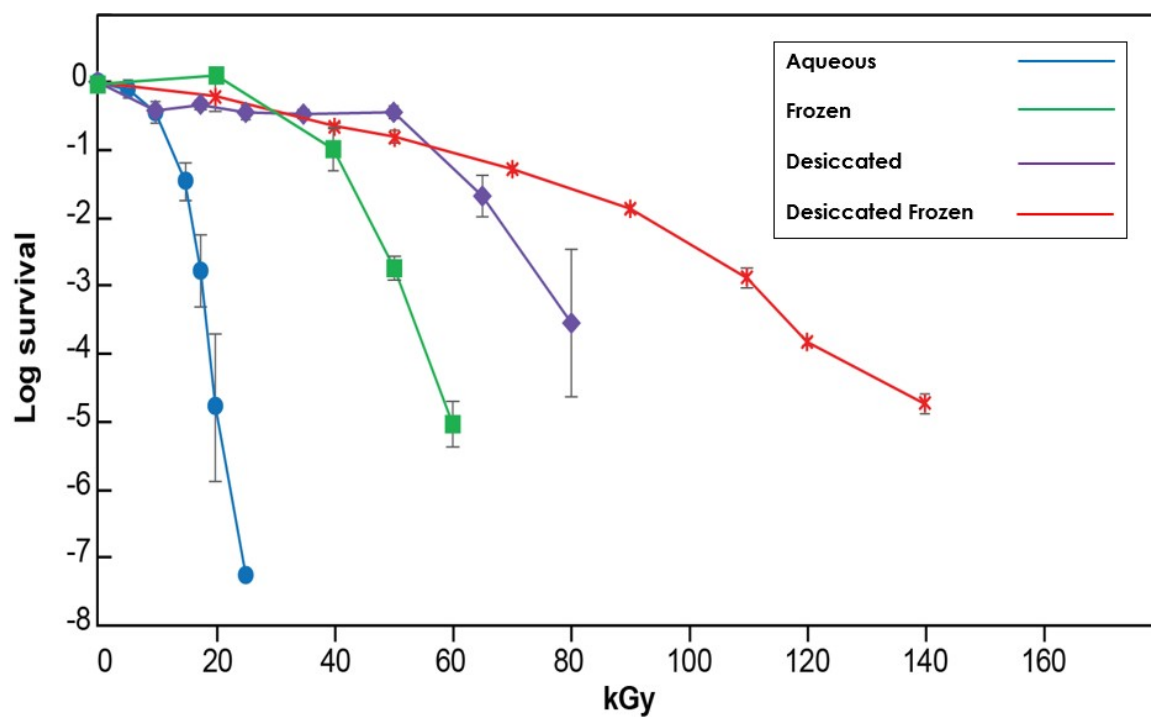


Figure 2. *D. radiodurans* gamma radiation survival under different environmental conditions.

Survival curves taken from samples of *D. radiodurans* culture that was either left in aqueous state and irradiated on wet ice (Aqueous); frozen to -80°C and irradiated on dry ice (Frozen); desiccated to dryness and irradiated on wet ice (Desiccated), or desiccated to dryness, frozen to -80°C, and irradiated on dry ice (Desiccated Frozen). Used with permission from Robert Volpe, *et al.* [94].

GOAL AND AIM OF THESIS

Humans are confronted with ionizing radiation every day [1] and have learned to harness it for a variety of purposes, including sterilization [3-5], medicine [9-11], and energy production [13]. However, understanding of the nature of radiation and its effects continues to change and evolve [19, 20, 22, 24, 47, 49, 58]. Significant research has gone into the diverse methods organisms use to survive radiation exposure, including enzymes and non-enzymatic antioxidants [35, 80-86]; fewer studies, though, have addressed one of the key sources of radiation-induced damage *in vivo*: water radiolysis. Some researchers have explored how changes in water content and form affect radiation damage in DNA or protein solutions *in vitro* [32, 88], while others have studied how these changes in water affect actual microorganism populations in specific commercial settings [89-92].

The focus of this study is to expand upon the work of Battista *et al.* [93], Volpe *et al.* [94], and Richmond *et al.* [95] to address the hypothesis that “a physiochemical environment that changes the water content or state of a microbe (e.g. by desiccation and/or deep freezing), without killing it, should increase the microbe’s survival to ionizing radiation.” The study accomplished this task by conducting a systematic analysis of gamma radiation survival of multiple microbial strains. Each strain was subjected to a standardized set of environmental modifications, including desiccant-facilitated drying and freezing at -80°C (**Fig. 3**), in order to test if the survival increases reported by others for *D. radiodurans* are specific to that strain alone, or applicable to all forms of microscopic life. Microorganisms with a variety of chromosome complexity, morphology, water content, and known radiation and desiccation resistance were chosen

to help identify unique cellular traits that can contribute to or hinder water's impact on radiation injury.

This study provides an experimental platform that can be expanded in breadth and depth to elucidate how ionizing radiation affects cells under a variety of potential environmental conditions, with practical applications in radiation sterilization techniques, space exploration, and numerous other fields of study.

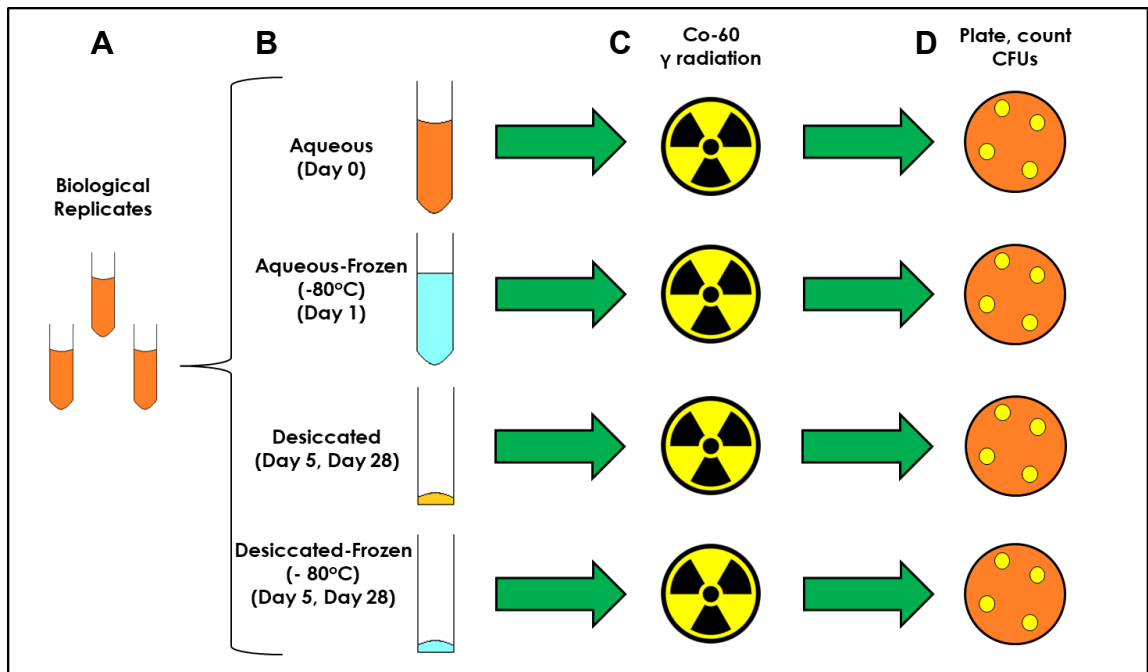


Figure 3. Experiment design outline. **A.** Biological replicates are grown overnight under optimal conditions. **B.** Replicates aliquoted to environmental condition cohorts (aqueous, aqueous-frozen, desiccated, desiccated-frozen). **C.** Cohorts exposed to Co-60 gamma radiation. **D.** Irradiated samples plated, and colony-forming units (CFUs) counted for analysis.

CHAPTER 2: Materials and Methods

Microorganisms used.

The bacteria and yeasts used in this study are listed in Table 1. They were obtained from the collections of Dr. Michael Daly (USU) (*E. coli*, *A. baumannii*, *L. plantarum*, *S. cerevisiae* EXF-6761, and *S. cerevisiae* EXF-6218) and Dr. Peter Setlow (UCONN) (*B. megaterium*, *B. thuringiensis*, and *B. subtilis*), and were chosen because they are whole genome-sequenced laboratory models with a range of resistance to desiccation and radiation to enable comparisons between strains (Table 1). Among the bacteria, only *Bacillus* species are capable of sporulation [96]; spores of the yeasts were not examined in this study because of time constraints.

Growth of microorganisms.

Optimal growth conditions for the microbes selected are well-developed and published [80, 96-99] as summarized in Table 2, using the media compositions described below and in Table 3:

- Luria-Bertani (LB) medium, Miller form: 1.0% (wt/v) tryptone, 0.5% yeast extract, 1.0% NaCl in deionized water (dH₂O)
- Tryptic Soy Agar (TSA): 4.0% manufacturer-prepared mixture in dH₂O
- Tryptic Soy Broth (TSB): 3.0% manufacturer-prepared mixture in dH₂O
- Lactobacilli MRS Broth: 5.5% manufacturer-prepared mixture in dH₂O
- Yeast Peptone Dextrose (YPD): 1.0% yeast extract, 2.0% peptone, 2.0% d-glucose in dH₂O

Table 1: Microorganisms used in this study

Category	Species	Strain	Genome Size (Mbp)	Literature D ₁₀ (kGy)	Characteristics/Reason Selected
Bacteria	<i>Escherichia coli</i>	K-12 (MG1655)	4.6	0.7	Classic model organism; ubiquitous in human microbiome; desiccation-sensitive, radiation-sensitive [47, 80]
	<i>Acinetobacter baumannii</i>	AB5075	3.97	Unknown	BSL-2; military-relevant pathogen; desiccation-resistant [100-102]
	<i>Lactobacillus plantarum</i>	ATCC 14917	3.24	0.47 - 0.7	Ubiquitous in foodstuffs, radiation-resistant [102, 103]
Yeasts	<i>Saccharomyces cerevisiae</i>	EXF-6761	10	3.5	Classic model organism; ubiquitous in nature; diploid; ionizing radiation-resistant [80]
	<i>Saccharomyces cerevisiae</i>	EXF-6218	14	0.8	Classic model organism; ubiquitous in nature; diploid; ionizing radiation-sensitive [80]
Bacterial Spores	<i>Bacillus megaterium</i>	QM B1551	5.1 + plasmids	3.0	Digenomic; reduced intracellular water (37-49% wt/wt) [104-108]
	<i>Bacillus thuringiensis</i>	Al Hakam	5.3	Unknown	Digenomic; reduced intracellular water (35-65%) [104-106, 109]
	<i>Bacillus subtilis</i>	PS533	4.2	2.0	Monogenomic; reduced intracellular water (35-65%) [104-106, 110, 111]

Agar plates for LB and MRS media were prepared with the addition of 1.5% agar; agar plates for YPD were prepared with 2.0% agar.

Culture inoculation and strain-specific growth conditions.

For the five vegetative microorganisms listed in Table 1, the strains were first cultured on agar plates yielding colony forming units (CFUs). Single colonies (one per replicate) were transferred to either 10 mL of appropriate medium (Table 2) in CentriStar™ sterile 50 mL centrifuge tubes (Corning®; Corning, NY; Catalog #430829), or 25 mL of medium in sterile 125 mL polycarbonate Erlenmeyer flasks with vent cap (Corning®; Catalog #431143), maintaining a 4:1 ratio of air to media in the container. The cultures were then incubated in an orbital shaker overnight at 200 rpm at optimal temperature (Table 2) for 18 hours. OD₆₀₀ readings of each replicate were then recorded using a DU® 730 Life Science UV/Vis Spectrophotometer (Beckman Coulter®; Fullerton, CA).

Culturing *Bacillus* species and isolation of spores.

Bacillus spores were prepared and purified in Dr. Setlow's lab by previously published methods [96, 112, 113]. Briefly, cells were grown at 37°C in LB medium (Table 3), then cultured on 2x Schaeffer's-glucose (SG) medium agar plates at 37°C for up to 5 days to induce sporulation. The resulting spores were then harvested and purified, including a final step of microcentrifugation through a 50% (wt/volume) buffer solution of Histodenz (a non-ionic density gradient medium) and water. The purified spores were

Table 2: Growth media used for microorganism cultures [80, 96-99]

Species	Strain	Medium Used	Incubation Temperature	Culture OD ₆₀₀ Readings
<i>Escherichia coli</i>	K-12 (MG1655)	Luria-Bertani (LB) Medium, Miller Form	37°C	3.70 - 4.01
<i>Acinetobacter baumannii</i>	AB5075	Tryptic Soy Agar (TSA), Tryptic Soy Broth (TSB)	37°C	2.69 - 3.00
<i>Lactobacillus plantarum</i>	ATCC 14917	Lactobacilli MRS Broth	37°C	3.65 - 3.72
<i>Saccharomyces cerevisiae</i>	EXF-6761	Yeast Peptone Dextrose (YPD)	30°C	4.17 - 5.21
<i>Saccharomyces cerevisiae</i>	EXF-6218	Yeast Peptone Dextrose (YPD)	30°C	4.36 - 4.41
<i>Bacillus megaterium</i>	QM B1551	Nanopure Water	N/A	N/A
<i>Bacillus subtilis</i>	PS533	Nanopure Water	N/A	N/A
<i>Bacillus thuringiensis</i>	Al Hakam	0.1% Tween in Nanopure Water	N/A	N/A

N/A: Not applicable

then suspended in nanopure water to prevent spore germination, with 0.1% Tween added for *B. thuringiensis* to prevent spore clumping (Tables 2 and 3). Samples were received frozen on dry ice at approximately 5×10^9 spores in 5 mL of nanopure water or Tween solution (1×10^9 spores/mL). The samples were thawed and diluted to 50 mL (1×10^8 spores/mL) in nanopure water, with 25 mL of the resulting suspension frozen for future study and 25 mL for testing.

Sterile technique.

All work with microorganisms (except *A. baumannii*, see below) was done under aseptic flame conditions. All media and equipment used were sterilized via autoclaving prior to use (temperature: 121°C; pressure: 22 pounds per square inch absolute (psia); duration: 40 minutes for media, 20 minutes for equipment).

Table 3: Growth media components

Medium	Contents	Notes
Luria-Bertani (LB) Medium, Miller Form	10 g Bacto™ Tryptone (Becton, Dickinson, and Co. (BD); Sparks, MD; Catalog #211705) 5 g Bacto™ Yeast Extract (BD; Catalog #212750) 10 g NaCl (Sigma-Aldrich; St. Louis, MO; Catalog #S3014-500G) 15 g Bacto™ Agar (BD; Catalog #214010) (for solid plates) 1.00 L dH ₂ O	None
Tryptic Soy Broth (TSB)	30 g Remel Tryptic Soy Broth (TSB) (Remel; Lenexa, KS; Catalog #R455054) 1.00 L dH ₂ O	Prepared per manufacturer's specifications
Tryptic Soy Agar (TSA)	40 g BBL™ Trypticase™ Soy Agar Modified (TSA II) (BD; Catalog #212305) 1.00 L dH ₂ O	Prepared per manufacturer's specifications
<i>Lactobacilli</i> MRS Broth	55 g Difco™ <i>Lactobacilli</i> MRS Broth (BD; Catalog #288130) 15 g Bacto™ Agar (for solid plates) 1.00 L dH ₂ O	Prepared per manufacturer's specifications
Yeast Peptone Dextrose (YPD)	10 g Bacto™ Yeast Extract 20 g Bacto™ Peptone (BD; Catalog #211677) 20 g d-Glucose (Sigma-Aldrich; Catalog #G8270-1KG) 20 g Bacto™ Agar (for solid plates) 1.00 L dH ₂ O	None
Nanopure Water	1.00 L H ₂ O purified by D11931 Nanopure Diamond Life Science UV/UF Water Purification System (Barnstead Lab Water Products; Lake Balboa, CA)	Used to prevent germination of spores
0.1% Tween in Nanopure Water	495 mL H ₂ O purified by D11931 Nanopure Diamond Life Science UV/UF Water Purification System 5 mL 10% Tween 20 Solution (Bio-Rad; Hercules, CA; Catalog #161-0781) 1x 500 mL PES vacuum filtration unit, 0.22µm filter (Stellar Scientific; Baltimore, MD; Catalog #TC-FIL-50022)	Used to prevent germination of spores; Tween used to prevent spore clumping in solution; sterilized by vacuum filtration

Aqueous and aqueous-frozen sample preparation.

300 μ L aliquots of culture or suspension were transferred to 1.5 mL tapered end Eppendorf Safe-Lock tubes (or CryoELITE[®] cryogenic vials (Wheaton Science Products; Millville, NJ; Catalog #W985852) for *A. baumannii*) for each replicate, condition, and gamma radiation dose studied. To avoid possible confounding factors and better simulate environmental conditions, no cryoprotectant was used during freezing. Aqueous-frozen samples were transferred to a -80°C freezer overnight for freezing.

Desiccated and desiccated-frozen sample preparation.

Two desiccator chambers, one for each condition (desiccated and desiccated-frozen), were loaded with 2 lbs of Drierite desiccant (W.A. Hammond Drierite Company; Xenia, OH; Catalog #21001) and hermetically sealed. Two Costar[®] Stripwell[™] 96-well microplates (Corning[®]; Catalog #9102) were loaded with sample from each replicate, aliquoting 25 μ L per well. To avoid possible confounding factors and better simulate environmental conditions, no lyoprotectant was used during desiccation. One 96-well plate, without lid, was placed into each desiccator chamber. The chambers were then closed and sealed with Parafilm[®] (Bemis[®]; Neenah, WI; Catalog #PM-996) and left at standard ambient conditions. Four days after the desiccators were loaded, one was transferred to a -80°C freezer, with the other left at ambient conditions. For the desiccation irradiations, one well was removed from each plate (ambient and frozen) for each replicate, condition, and radiation dose studied. These wells were then loaded into 2.0 mL Eppendorf[®] Safe-Lock tubes (Eppendorf[®]; Catalog #022363352) (or CryoELITE[®] cryogenic vials for *A. baumannii*).

Biosafety Level 2 (BSL-2) handling methods for *A. baumannii* AB5075.

Additional safety precautions were taken when working with *A. baumannii* samples. Proper personal protection equipment (PPE) was worn while handling *A. baumannii* and associated equipment. All *A. baumannii* work was done within an Interceptor® INT-1100 Class II A2 biological safety cabinet (Kewaunee® Scientific Corporation; Statesville, NC). All non-biological materials were decontaminated with ultraviolet C (UVC) radiation inside the cabinet before starting work. Screw-top cryogenic vials and agar plates were sealed with Parafilm® prior to transporting samples outside of the cabinet. All experimental waste was decontaminated with a 15% v/v bleach/water solution, then UVC, prior to disposal in biohazardous waste containers for autoclaving. Disposable air-tight containers were used as desiccator chambers for *A. baumannii*. The containers and Drierite were disposed as biohazardous waste upon termination of the *A. baumannii* experiment series. Incubators and refrigerators specifically designated as BSL-2 were used to hold *A. baumannii* culture and samples.

Irradiation schedule and method.

Samples were irradiated according to the following schedule:

- Day 0 (sample preparation day): Aqueous
- Day 1: Aqueous-frozen
- Day 5: Desiccated (ambient) and desiccated-frozen
- Day 28: Desiccated (ambient) and desiccated-frozen

Aqueous and desiccated samples were stored and irradiated on wet ice during the irradiation, while aqueous-frozen and desiccated-frozen samples were stored and irradiated on dry ice (-79°C). Gamma irradiations were performed in a J. L. Shepard and Associates Model 109 Co-60 irradiator (dose rates between 91.3 - 106.2 Gy/min), maintained by USU School of Medicine. For each replicate and condition, one sample was left unexposed to radiation (non-irradiated control).

Serial dilution plating.

Using existing survival values for each microorganism (Table 1), estimates were made regarding probable ranges of survival at each radiation dose and condition. Aliquots were then taken from each replicate, dose, and condition sample for dilution and study. For aqueous and aqueous-frozen, aliquots were taken directly from the samples; for desiccated and desiccated-frozen, well contents were resuspended in 250 µL of media to provide enough volume for study. Samples were diluted serially using media by factors of 10, and the dilutions estimated to have countable CFUs were plated on agar plates (100 µL of sample per plate). The plates were then incubated for up to 48 hours at optimal temperature (Table 2) and resulting CFUs were counted for each plating.

Single-plate spot dilution survival assay.

A Corning® 96-well assay plate (Corning®; Catalog # 3788) was prepared for each condition and replicate to conduct serial dilutions. Irradiated samples were placed in the first row (50 µL for aqueous and aqueous-frozen samples, 25 µL for desiccated and desiccated-frozen samples resuspended in 25 µL of media). The samples were then

diluted by a factor of 10 on each following row, to a final dilution of 10^{-7} . Using a multichannel pipette, 5 μL of each sample across each row were plated simultaneously on an agar plate so that each column on the plate represented a single dilution factor. These plates were then incubated for up to 48 hours, and the resulting plates with CFUs were photographed. The NIH ImageJ software package [114] was then used with the plate photographs to facilitate manual counting of CFUs for each dose at each dilution.

***Bacillus* strains spore counts.**

Irradiated samples from all four conditions were placed in sample boxes on dry ice and shipped to Dr. Setlow's lab for analysis. Once received, samples were serially diluted by factors of 10 in nanopure water to a final dilution of 10^{-6} . 10 μL of each dilution was spotted in duplicate on LB agar plates (10 $\mu\text{g/mL}$ Kanamycin added for *B. subtilis*). Desiccated and desiccated-frozen samples were suspended in 250 μL of nanopure water prior to dilution. Spotted plates were incubated 18 hours at 30°C and then at 37°C until colony counts were stable.

Data analysis.

CFU counts for all organisms were stored in human-readable spreadsheet files. These files were read into a custom Python script utilizing the pandas module [115] to process the data. The script normalized the CFU counts to the initial concentrations (aqueous non-irradiated control) for each organism, then further normalized the ratios to the non-irradiated control for each condition set. The resulting ratios were then $\log_{10}$ converted to create log survival values, and these values were averaged for each dose in

each condition. Next, GraphPad Prism 8 was used to perform quadratic-term regression on each data set using ordinary least squares and determine the statistical significance of differences between survival curves of the same organism using the B1 and B2 coefficient terms of each curve. Quadratic-term regression was used to model the characteristic “shoulder” seen in survival curves for organisms with high radiation resistance with the smallest order polynomial possible to avoid “over-fitting” [116, 117]. This model method is commonly used in clinical settings to determine radiotherapy doses needed to achieve specific cancer cell death levels [118]. Dose values (x) were interpolated for the -1 (D_{10} , 10% survival) and -5 ($D_{0.001}$, 0.001% survival) log survival values (y) of each curve for comparison. Non-irradiated log survival values were also interpolated from the curves normalized only by initial concentration to compare the effects each condition put on its cohort separate from ionizing radiation.

Outliers, resolution, and statistical adjustments.

For several organisms and conditions, data points at high radiation doses tended to form a tail to the regression curves. These values shared a trait in that the only plate reads that could be used for analysis came from the most undiluted samples available (10^0 for single plate spot dilution assays of all conditions and serial dilution platings of aqueous and aqueous-frozen irradiations; 10^{-1} for serial dilution platings of desiccated and desiccated-frozen radiations). Assuming that these readings could represent a limit of precision for the two plating methods, data values that met these criteria were removed for data analysis. For conditions that only had two to three data values, however, the high-radiation readings were retained for analysis. A complete data analysis done with

all data values in all sets can be found in the Supplemental Information section for comparison.

Also, the regression curves for several organism/condition sets had positive B2 (x^2) coefficients, which would predict that at extremely high radiation doses, survival would increase. To account for this discrepancy, an additional analysis was performed on all applicable data sets where the regression model was constrained to generate a B2 coefficient less than 0, which can be found in the Supplemental Information section.

CHAPTER 3: Results

BACTERIA

Escherichia coli

The pilot study of this research into the effects of desiccation and freezing on radiation survival was with *E. coli* K-12 (MG1655), chosen for its regular use as a model organism, ubiquitous presence in the human microbiome, and documented desiccation and radiation sensitivity [47, 80]; and was performed in two phases. The first phase focused on the effects of long-term (weeks) desiccation on *E. coli* gamma radiation survival, with dried cells collected on Days 14 and 28 for samples desiccated at either ambient conditions (18-23°C) or -80°C. The second phase focused on the four primary physicochemical conditions (aqueous, aqueous-frozen, desiccated, desiccated-frozen). Growth of cells and desiccation treatments for both phases were conducted under the same conditions as detailed in Methods, achieving similar OD₆₀₀ readings and growth phase for cultures in each study. Cell survival for both phases was determined using serial dilution CFU assays (Methods). Quadratic regression curves derived from the data had adjusted R² values between 0.998 and 0.987 (**Sup. Fig. 1A**).

The survival curves (**Fig. 4A**) demonstrate significant gamma radiation survival improvement for *E. coli* cells that were either desiccated or frozen. The D₁₀ dose for *E. coli* increased from 0.52 in aqueous condition to 0.69 kGy when samples were deeply frozen and to 1.45 kGy when they were desiccated (**Sup. Fig. 1B**). The relative survival increases for desiccation and freezing remained very similar at doses yielding greater

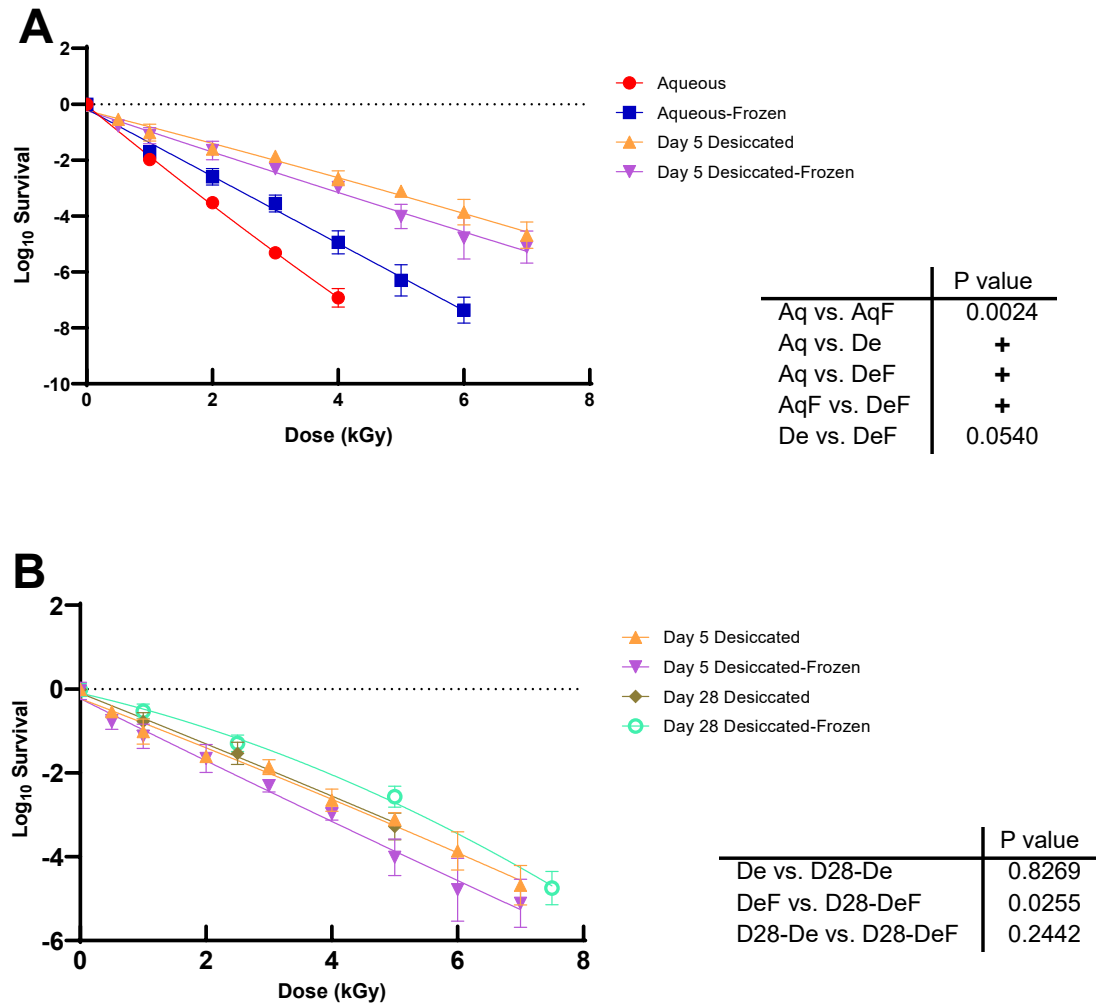


Figure 4. *E. coli* survival to gamma radiation under different physicochemical conditions. A. *E. coli* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *E. coli* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

reductions in survival ($D_{0.001}$) (**Sup. Fig. 1C**). There was no apparent synergy between the effects of freezing and desiccation at the doses measured (**Fig. 4A**); indeed, at lower doses (D_{10} range), freezing appeared to inhibit the survival benefit of desiccation (**Sup. Fig. 1B**), but this trend did not meet significance. As desiccation durations increased, the only significant change noted was that desiccated-frozen samples had greater gamma

radiation survival on Day 28 than Day 5 (**Fig. 4B**). In short, freezing had no significant effect on desiccated *E. coli* gamma radiation survival at any time point studied.

In non-irradiated cultures, freezing *E. coli* had little impact on survival; however, desiccation noticeably reduced *E. coli* survival, as reported previously [72]. These losses continued to increase as the desiccation progressed. Freezing desiccated samples appeared to improve survival slightly, but the significance of this trend is uncertain (**Sup. Fig. 2**).

Constraining the *E. coli* aqueous regression curve to a negative B2 coefficient only slightly modified the resulting curve (-0.001 to the adjusted R^2 , no change in D_{10} , +0.02 kGy in $D_{0.001}$), with no appreciable change in the results (**Sup. Fig. 3, 4**). When analyzing all data points from the aqueous, aqueous-frozen, and desiccated-frozen samples, the difference between Day 5 desiccated and desiccated-frozen samples became significant, but all other results remained valid (**Sup. Fig. 5, 6**). Neither alternate analysis method had a noticeable impact on *E. coli* non-irradiated survival to desiccation or freezing (**Sup. Fig. 7, 8**).

Acinetobacter baumannii

A. baumannii AB5075 was used in this study because it is a desiccation-resistant BSL-2 organism known to have infected several U.S. military servicemembers during recent deployments to Iraq [100, 101]. This experiment series was conducted under BSL-2 conditions using the single-plate spot dilution survival assay to measure survival while limiting biohazardous waste production (Methods). This method also provided 24-hour turnaround on CFU counts, revealing on Day 6 that the Day 5 desiccated samples needed more irradiation to reach last survivors (**Sup. Fig. 9C, D**). Additional desiccated

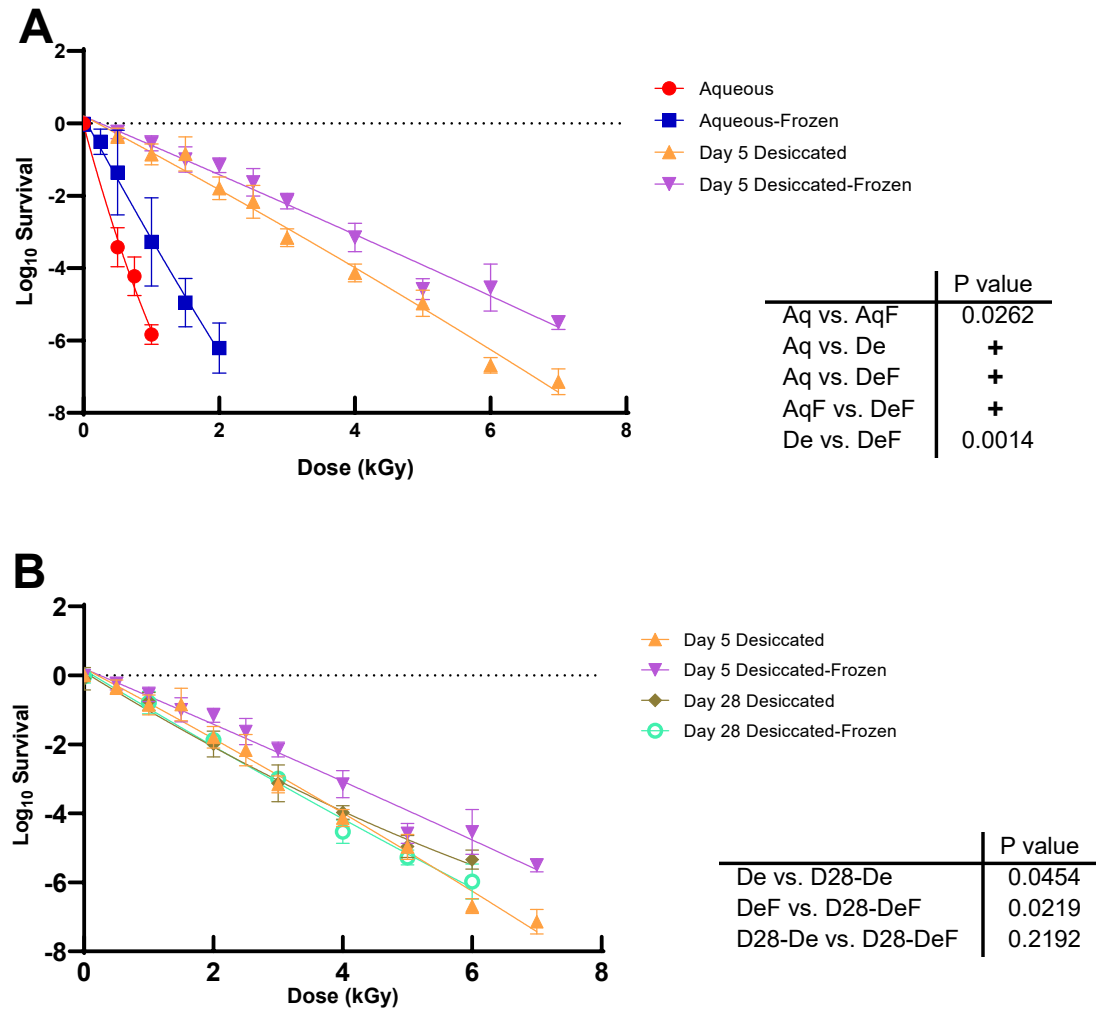


Figure 5. *A. baumannii* survival to gamma radiation under different physicochemical conditions. A. *A. baumannii* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *A. baumannii* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from two replicates.

samples were taken on Day 6 and irradiated at greater doses (Sup. Fig. 9C, D), with the resulting data converted to represent Day 5 values (Sup. Fig. 10). With the conversion performed, regression curves from the resulting replicate data had adjusted R^2 values between 0.973 and 0.992 (Sup. Fig. 1A).

As with *E. coli*, *A. baumannii* gamma radiation survival significantly improved when samples were either frozen or desiccated (**Fig. 5A**), with desiccation once again providing the greater survival benefit (D_{10} of 0.34 kGy for aqueous-frozen and 1.19 kGy for Day 5 desiccated samples versus 0.14 kGy for aqueous) (**Sup. Fig. 1B**). These survival benefits trended downward, however, when survival rates reached $D_{0.001}$ (**Sup. Fig. 1C**). There was significant synergy between desiccation and freezing (**Fig. 5A**), with a similar downward trend between D_{10} and $D_{0.001}$ values (**Sup. Fig. 1C**). When desiccations reached the Day 28 mark, both desiccated and desiccated-frozen samples had a significant decrease in gamma radiation survival compared to the Day 5 samples (**Fig. 5B**); however, this decrease was more pronounced for D_{10} values versus $D_{0.001}$ values (**Sup. Fig. 1B, C**).

There were some unexpected data noted for *A. baumannii* non-irradiated desiccation and freezing population losses. The aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen samples all had projected non-irradiated sample concentrations higher than aqueous, up to a 0.4-log increase for Day 5 desiccated versus aqueous samples (**Sup. Fig. 2**). Day 28 desiccated and desiccated-frozen samples had less non-irradiated survivors than their corresponding Day 5 samples (**Sup. Fig. 2**). As *A. baumannii* was the only cluster-forming organism studied [119], the most likely cause of elevated non-irradiated populations in non-aqueous samples would be cluster agitation occurring during freezing and desiccation, splitting one cluster CFU into multiple CFUs. This discrepancy does not affect survival curve findings, however, as all curves were normalized to their non-irradiated dose value.

Constraining *A. baumannii* survival curves to negative B2 terms altered several results. Modeling accuracy decreases negated the significance of differences between aqueous and aqueous-frozen samples, as well as Day 5 and Day 28 desiccated samples (**Sup. Fig. 11**). This constraint also generated a negative D₁₀ value for the aqueous condition (-0.09 kGy) (**Sup. Fig. 3**), creating a new logic flaw that brings the other model findings into question. Analyzing all *A. baumannii* data points resulted in poorer curve fits for most curves (**Sup. Fig. 5**), making the aqueous and aqueous-frozen difference non-significant (**Sup. Fig. 12**). This analysis also shifted the aqueous curve lower compared to the other curves, resulting in a decrease in D₁₀ value (**Sup. Fig. 12**) and increased relative non-irradiated desiccation- and freezing-survival across all other conditions (**Sup. Fig. 8**).

Lactobacillus plantarum

Lactobacillus plantarum ATCC 14917 was studied because of its common presence in foodstuffs and consumable products, as well as its documented radiation resistance despite its lack of antioxidant enzymes [103]. While performing this experiment series per the steps described in Methods, both the desiccated and desiccated-frozen samples experienced substantial population loss prior to Day 5. Because of this population loss, no analysis could be performed on any desiccated samples. As for the aqueous and aqueous-frozen samples, the three replicates studied using serial plate dilution generated regression models that achieved adjusted R² values of 1.000 and 0.999, respectively (**Sup. Fig. 1**).

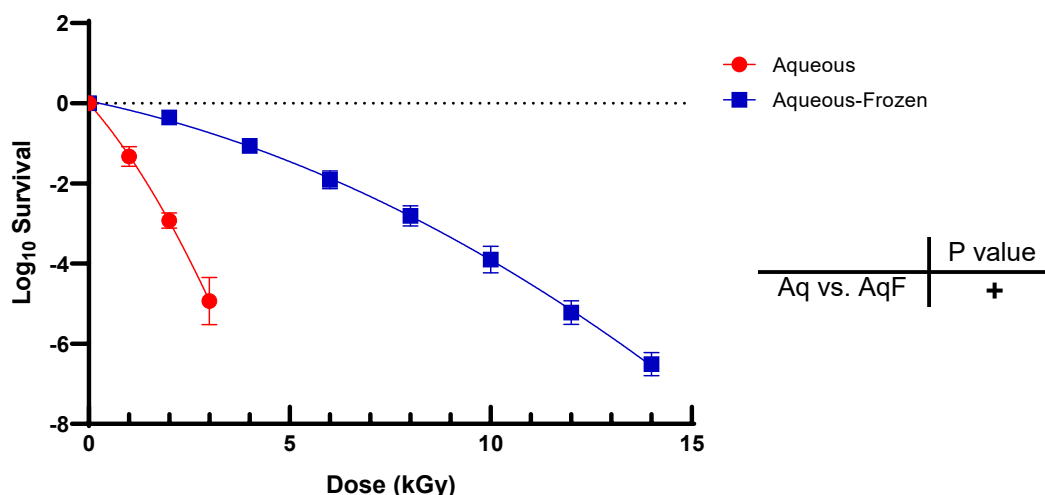


Figure 6. *L. plantarum* survival to gamma radiation under different physicochemical conditions. *L. plantarum* survival curves for aqueous and aqueous-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

Freezing *L. plantarum* cultures improved gamma radiation survival significantly, increasing its D_{10} from 0.79 kGy to 3.78 kGy (**Fig. 6, Sup. Fig 1**) and its $D_{0.001}$ from 3.03 kGy to 11.76 kGy (**Sup. Fig 1**). Freezing also decreased non-irradiated culture population by a log factor of 0.2 (**Sup. Fig. 2**). With both curves having negative B2 values and all data points meeting inclusion criteria for analysis, no additional analyses were performed for this data.

YEASTS

Saccharomyces cerevisiae EXF-6761

Saccharomyces cerevisiae EXF-6761 was chosen for this study due to its regular use as a model organism, ubiquitous presence in nature, and its high documented resistance to radiation [80]. This experiment series was conducted with no noted deviations from listed methods. Replicates were studied using the serial dilution plating

method. Quadratic regression curves derived from the data had adjusted R^2 values between 0.998 and 0.896 (**Sup. Fig. 1**).

EXF-6761 survival curves (**Fig. 7A**) indicate that freezing and desiccating samples significantly improved gamma radiation survival, with D_{10} values (7.06 kGy and 7.38 kGy, respectively) greater than aqueous (3.68 kGy) (**Sup. Fig. 1B**). The relative survival benefit trended upward at $D_{0.001}$ doses (18.24 kGy and 21.14 kGy versus 8.15 kGy, respectively), with freezing and desiccation also demonstrating significant synergy at $D_{0.001}$ doses (26.96 kGy) (**Sup. Fig. 1C**).

As desiccation duration increased, freezing radiation survival benefits diminished for EXF-6761. Whereas Day 5 desiccated and desiccated-frozen samples were significantly different (**Fig. 7A**), Day 28 desiccated and desiccated-frozen samples were not (**Fig. 7B**). The Day 28 desiccated and desiccated-frozen samples also shared similar D_{10} and $D_{0.001}$ value patterns (**Sup. Fig. 1B, C**). Of note, the Day 28 samples trended towards improved survival compared to the Day 5 desiccated sample as radiation doses increased; however, the differences between the three curves were not significant (**Fig. 7B**).

Freezing and desiccating non-irradiated culture had moderate and cumulative effects on EXF-6761 survival but remained below a 1-log survival difference even when combined (**Sup. Fig. 2**). Longer (28-day) desiccations had no discernable survival difference between each other or with Day 5 desiccated-frozen samples (**Sup. Fig. 2**).

No B2 constraint analysis was needed for EXF-6761. When all aqueous, aqueous-frozen, and Day 28 desiccated and desiccated-frozen data points were analyzed,

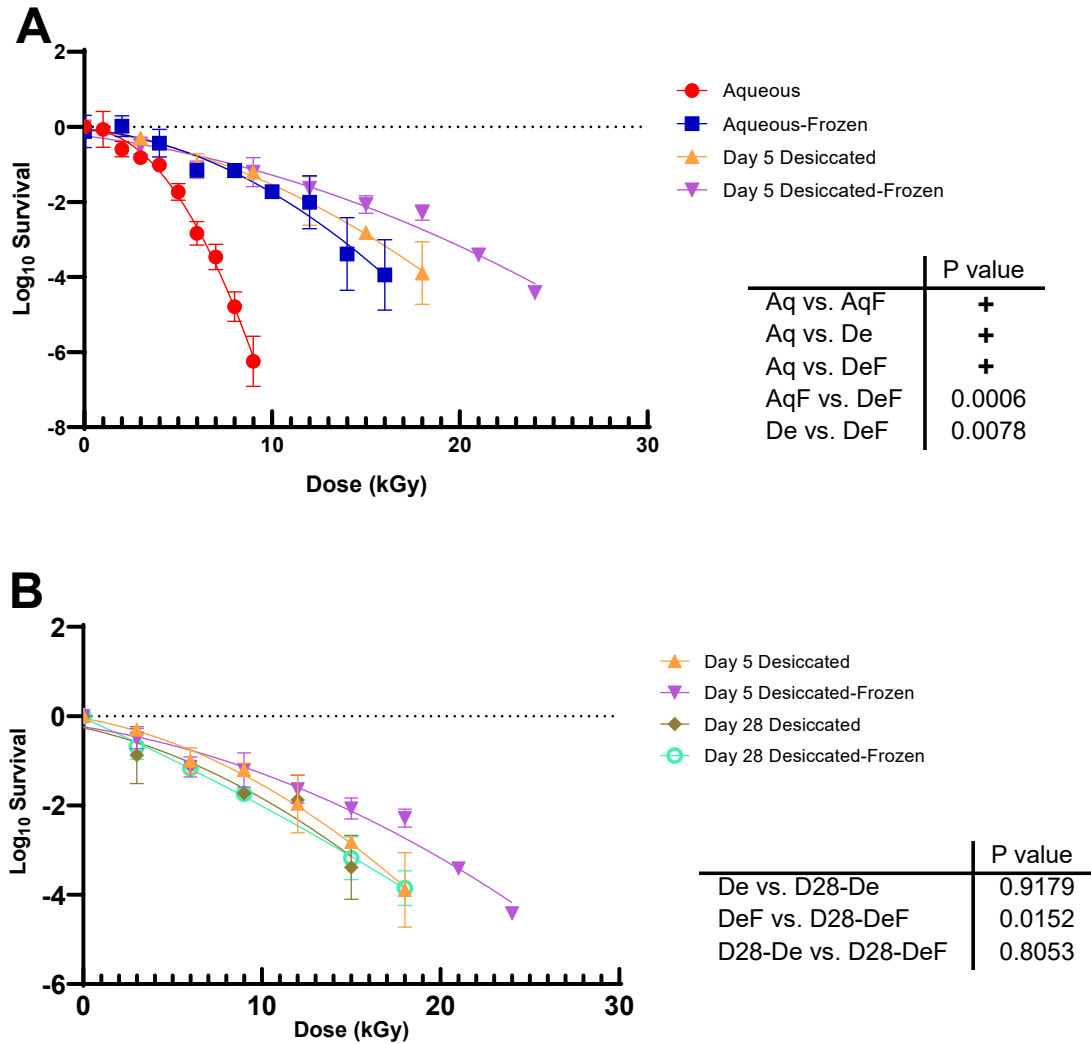


Figure 7. *S. cerevisiae* EXF-6761 survival to gamma radiation under different physicochemical conditions. **A.** *S. cerevisiae* EXF-6761 survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *S. cerevisiae* EXF-6761 survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

there were minor variations in curve values (Sup. Fig. 5), but no differences in significance of comparisons or data trends (Sup. Fig. 13).

***Saccharomyces cerevisiae* EXF-6218**

Saccharomyces cerevisiae EXF-6218 was chosen for this study due to its regular use as a model organism, ubiquitous presence in nature, and its low documented resistance to radiation relative to *S. cerevisiae* EXF-6761 [80]. This experiment series was performed twice. During the first series using the serial dilution plating method (Methods), there were significant delays in plating and analyzing the desiccated and desiccated-frozen samples. When the resulting data appeared much different than data from other vegetative organisms (**Sup. Fig. 14**), there were concerns that prolonged (four months) storage at -80°C had compromised the desiccated samples. A second experiment series was conducted for EXF-6218 using the single-plate spot dilution survival assay method (Methods) to expedite data collection and prevent possible irradiated sample degradation due to cold storage (**Sup. Fig. 15**). The regression curves derived from the second series had adjusted R^2 values mostly between 0.908 and 0.982; however, the Day 28 desiccated samples had a value of 0.690, indicating a poorer fit to experimental data (**Sup. Fig. 1A**).

Freezing significantly improved gamma radiation survival in EXF-6218 (**Fig. 8A**); this survival benefit also appears to increase at higher radiation doses ($D_{0.001}$ values of 12.46 kGy and 4.54 kGy for aqueous-frozen and aqueous, respectively, versus D_{10} values of 3.27 kGy and 1.71 kGy) (**Sup. Fig. 1B, C**). Desiccation also increased gamma radiation survival in EXF-6218 (D_{10} value 2.03 kGy); however, the Day 5 desiccated survival curve model could not extrapolate a $D_{0.001}$ value for comparison (**Fig. 8A, Sup. Fig. 1B, C**). Indeed, the models generated for Day 5 desiccated and desiccated-frozen samples have strong positive B2 terms, making it difficult to accurately compare these

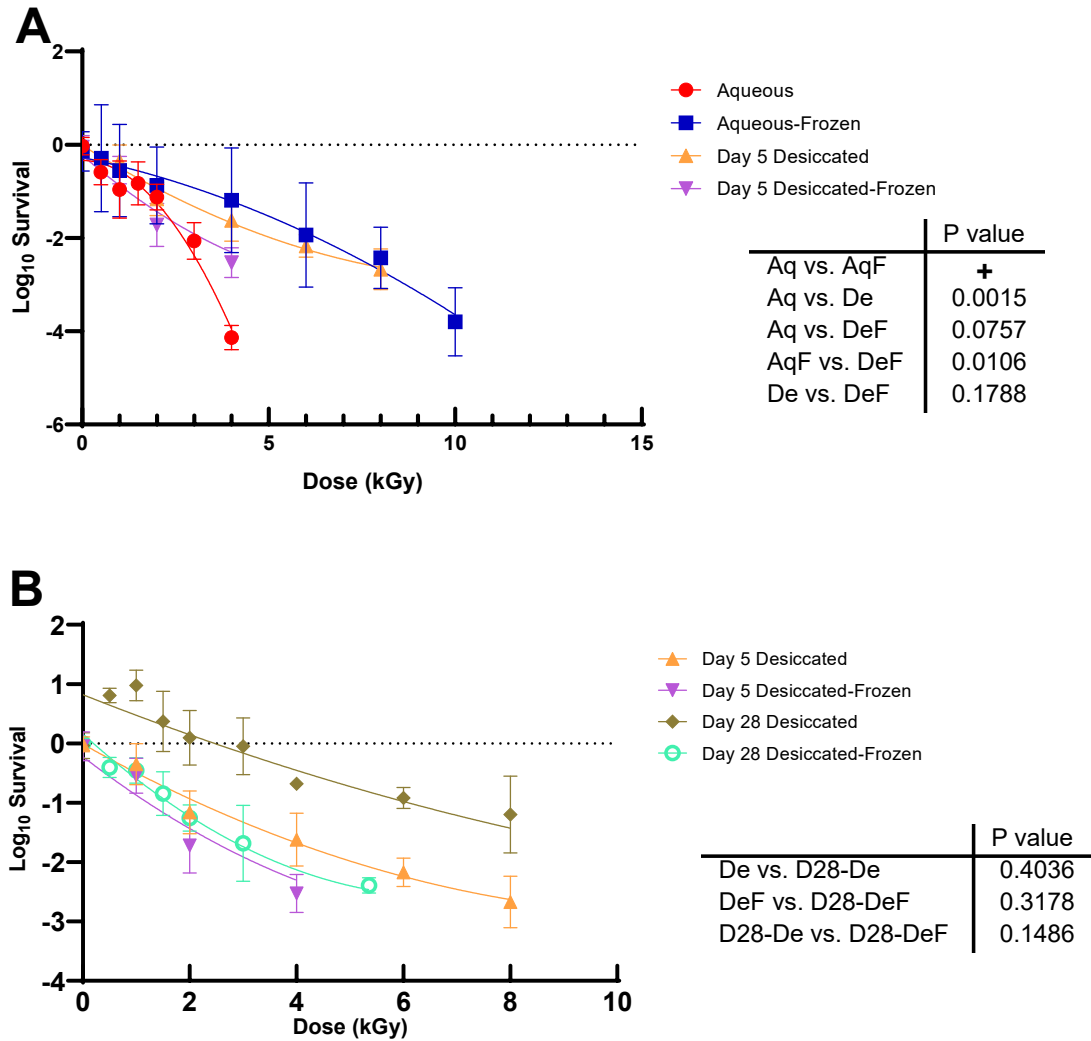


Figure 8. *S. cerevisiae* EXF-6218 survival to gamma radiation under different physicochemical conditions. **A.** *S. cerevisiae* EXF-6218 survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *S. cerevisiae* EXF-6218 survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from two replicates.

curves with the aqueous and aqueous-frozen curves (**Fig. 8A**). Although the Day 5 desiccated-frozen curve is consistently below the Day 5 desiccated curve, significance cannot be given to this observation (**Fig. 8A**).

The Day 28 desiccated and desiccated-frozen survival curves also shared features from the Day 5 survival curves (**Fig. 8B**). This design prevented $D_{0.001}$ value extrapolation (**Sup. Fig. 1C**). For D_{10} values, there was an almost 3-fold increase between Day 28 and Day 5 desiccated samples; however, there was no significance for any of the Day 5 or Day 28 desiccated sample comparisons (**Fig. 8B**). Also of note, the Day 28 desiccated curve had data points that went above the x axis, indicating that at these gamma radiation doses, EXF-6218 survival increased rather than decreased (**Fig. 8B**). Yeast sporulation due to nutrient loss from desiccation [120] is one explanation of these divergent survival curve findings. Ascospore tetrad release, triggered either internally or through low-level gamma radiation damage, could increase CFU counts from one diploid cell to four haploid cells, confounding CFU counts and analyses. Day 28 desiccated variations would then be the result of additional time and optimum temperature for spore formation relative to the other desiccated samples. A microscopic study of EXF-6218 desiccation at multiple time points would be an effective method to validate this theory.

Desiccation had a dramatic impact on EXF-6218 non-irradiated populations. While freezing produced a 0.3-log decrease, 5 days of desiccation produced a 1.1-log decrease, while 28 days produced a 2.1-log decrease. For short desiccations, freezing appears to increase population loss; for long desiccations, freezing may reduce population loss (**Sup. Fig. 2**).

B2 term constraint analysis of EXF-6218 reduced the survival curve fits for Day 5 desiccated and desiccated-frozen samples, as well as Day 28 desiccated-frozen samples (**Sup. Fig. 3A**). There was also a loss of significance in the aqueous versus Day 5

desiccated comparison and an inability to calculate the Day 5 desiccated versus desiccated-frozen or Day 5 versus Day 28 Desiccated comparisons (**Sup. Fig. 16A, B**). D_{10} comparisons followed a similar trend between the two sets of analyses (**Sup. Fig. 3B, C**).

Analyzing all EXF-6218 data points enhanced the positive B2 term of all desiccated curves (**Sup. Fig. 17A, B**) and improved the fit of the aqueous and Day 5 desiccated and desiccated-frozen curves; however, it decreased the fit of the Day 28 desiccated-frozen curve (**Sup. Fig. 5A**). The aqueous and Day 5 desiccated-frozen, Day 5 desiccated and desiccated-frozen, and Day 28 desiccated and desiccated-frozen comparisons also gained significance (**Sup. Fig. 17A, B**). However, there was little variation in the relative D_{10} or $D_{0.001}$ differences between this analysis and the original analysis (**Sup. Fig. 5B, C**). There was also little variation in non-irradiated population loss due to desiccation and freezing across all three analysis methods (**Sup. Fig. 7, 8**).

Comparing this experiment series to the initial EXF-6218 series (**Sup. Fig. 14**), the aqueous and aqueous-frozen curves both had similar shapes, significant difference, and trend for increasing gamma radiation resistance in aqueous frozen samples as radiation dose increases. However, the Day 5 desiccated and desiccated-frozen curves both had negative B2 terms, resulting in a dramatically different shape compared to similar curves from the second series (**Sup. Fig. 14**).

BACTERIAL SPORES

Bacillus megaterium QM B1551.

Sporulated *Bacillus megaterium* QM B1551 was selected for study because of its lower intracellular water content relative to the vegetative organisms in this study as well as its digenomic genetic content [104-106]. The experiment series was conducted without any noted deviations from listed methods. The survival curves derived from the experimental data had adjusted R^2 values from 0.963 to 1.000 for all samples except Day 28 desiccated and desiccated-frozen (**Sup. Fig. 1**). For these samples, there were not enough data points available to create a regression model. Indeed, for the Day 28 desiccated samples, there were no survivors noted in any sample (**Sup. Fig. 2**).

The *B. megaterium* spore aqueous, aqueous-frozen, and Day 5 desiccated-frozen curves were very similar in shape, P-values, and $D_{10} / D_{0.001}$ values (**Fig. 9A, Sup. Fig. 1B, C**). The Day 5 desiccated curve had D_{10} (1.57 kGy) and $D_{0.001}$ (5.46 kGy) values that were lower than aqueous values (2.56 kGy and 9.28 kGy, respectively) (**Sup. Fig. 1B, C**); however, this difference did not meet statistical significance (**Fig. 9A**). This indicates that freezing alone does not have any effect on gamma radiation survival in *B. megaterium* spores, while desiccation may make the organism more radiosensitive, with more study required to verify. This also indicates that any loss in gamma radiation survival caused by desiccation can be attenuated by freezing.

Because no regression models could be built for Day 28 desiccated or desiccated-frozen samples, no comparisons can be made regarding desiccation duration for *B. megaterium* spores (**Fig. 9B**).

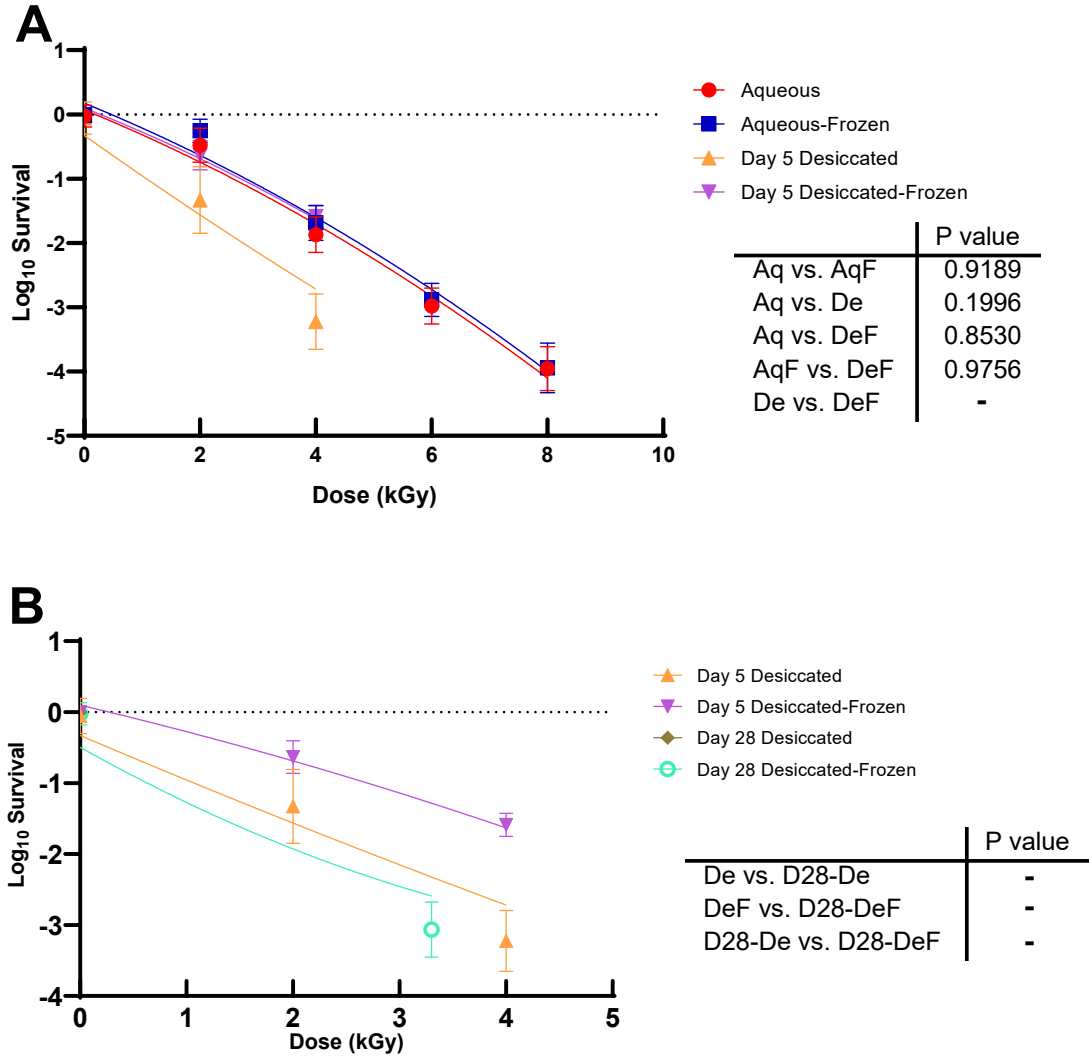


Figure 9. *B. megaterium* survival to gamma radiation under different physicochemical conditions. **A.** *B. megaterium* spore survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. megaterium* spore survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: unable to calculate. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

Freezing *B. megaterium* spores had little to no impact on non-irradiated survival, while desiccation for 5 days resulted in a 1.7-log loss in population. 28 days of desiccation, however, resulted in no observable spores even for non-irradiated samples. Once again, freezing seemed to mediate desiccation effects on survival, maintaining *B. megaterium* spore population levels at or above Day 5 desiccated levels (Sup. Fig. 2).

No B2-constraint analysis was needed for *B. megaterium* spores. When all available data points were analyzed, the aqueous and aqueous-frozen sample curves had improved fit, while the Day 5 desiccated-frozen curve had worse fit. The Day 28 desiccated and desiccated-frozen samples still did not have enough data points to generate regression curves (**Sup. Fig. 18**). As for non-irradiated population losses, there were only slight variations between the two assessment methods, with no changes to observed trends (**Sup. Fig. 5**).

Bacillus thuringiensis

Like *B. megaterium*, sporulated *Bacillus thuringiensis* Al Hakam was selected for study because of its lower intracellular water content relative to the vegetative organisms in this study as well as its digenomic genetic content [104-106]. The experiment series was conducted without any noted deviations from listed methods. The survival curves derived from the experimental data had adjusted R^2 values from 0.962 to 1.000 for all samples except Day 28 desiccated (**Sup. Fig. 1**). For this sample set, there were not enough data points available to create a regression model.

As with *B. megaterium*, the aqueous, aqueous-frozen, and Day 5 desiccated-frozen curves for *B. thuringiensis* spores had very similar shapes, p-values, and D_{10} / $D_{0.001}$ values (**Fig. 10A, Sup. Fig. 2**). Also, like *B. megaterium*, the Day 5 desiccated curve trended lower than the three curves mentioned above, with D_{10} (1.48 kGy) and $D_{0.001}$ (4.85 kGy) values less than aqueous values (2.51 kGy and 9.83 kGy), but this trend was not statistically significant (**Fig. 10A, Sup. Fig. 2**). The only statistically significant survival difference was between Day 5 desiccated and desiccated-frozen samples (**Fig.**

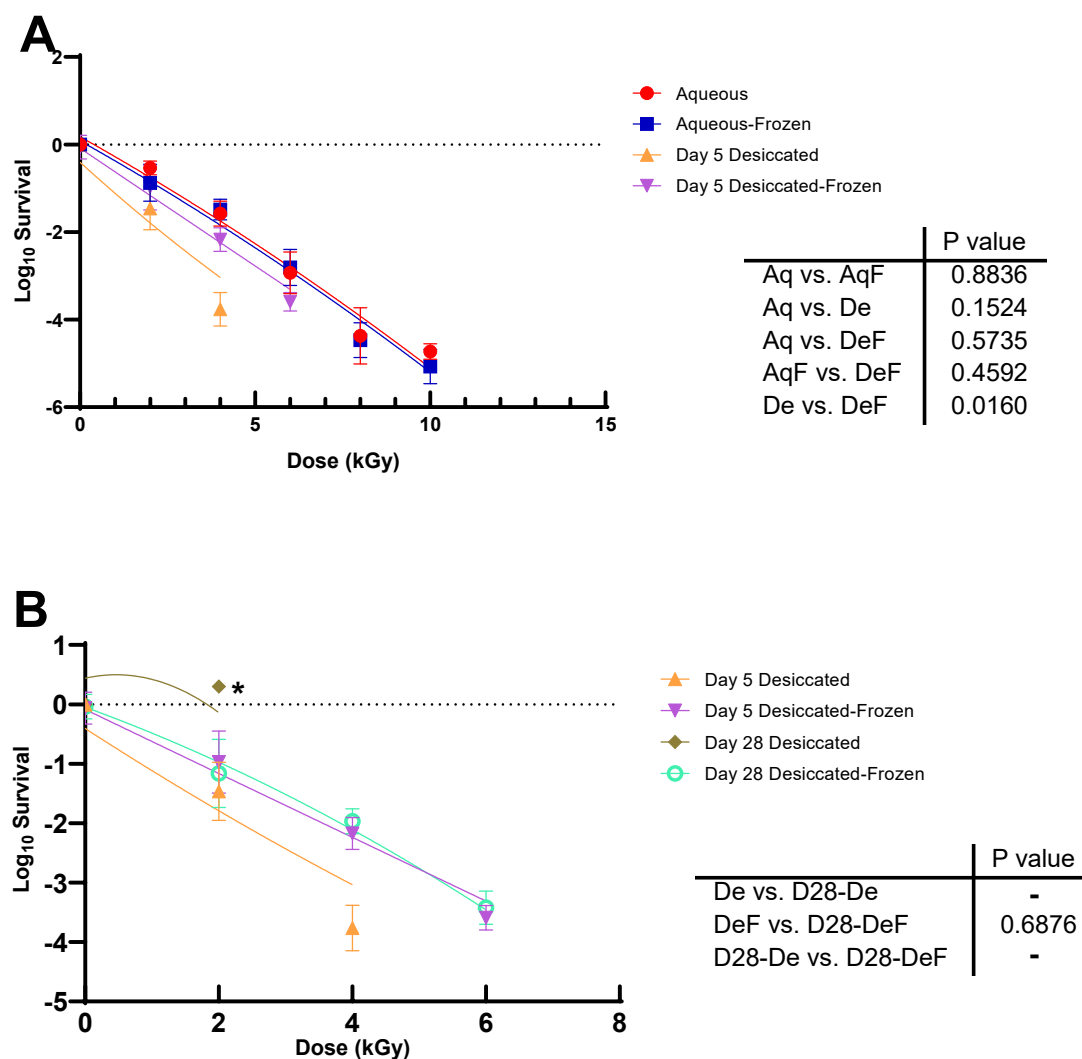


Figure 10. *B. thuringiensis* survival to gamma radiation under different physicochemical conditions. **A.** *B. thuringiensis* spore survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. thuringiensis* spore survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: unable to calculate; *: single reading at this data point. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

10A). Over time, there was no significant difference in survival between Day 5 and Day 28 desiccated-frozen samples (Fig. 10B).

When considering freezing and desiccation effects on non-irradiated *B.*

thuringiensis spores, freezing caused a 0.3-log decrease in population, while desiccation

caused a 1.0-log decrease. Freezing with desiccation appeared to improve survival compared to desiccation alone (**Sup. Fig. 2**). As desiccation duration increased, unfrozen sample populations declined rapidly, similar to *B. megaterium* (**Sup. Fig. 2**). Interestingly, Day 28 desiccated-frozen samples had less population loss than Day 5 desiccated-frozen samples (**Sup. Fig. 2**).

No B2-term constraint analysis was needed for *B. thuringiensis* spores. As for the all-points analysis, there were no noticeable changes in either gamma radiation resistance or non-irradiated survival findings that met statistical significance (**Sup. Fig. 5, Sup. Fig. 19**).

Bacillus subtilis

Sporulated *Bacillus subtilis* PS533 was selected for study because of its lower intracellular water content relative to the vegetative organisms in this study as well as its monogenomic genetic content [104-106]. The experiment series was conducted without any noted deviations from listed methods. The survival curves derived from the experimental data had adjusted R² values from 0.962 to 1.000 for most samples, with the Day 28 desiccated-frozen sample set having a value of 0.854 (**Sup. Fig. 1A**).

Only freezing had a statistically significant difference in survival compared to aqueous *B. subtilis* spores (**Fig. 11A**). The D_{0.001} values (7.85 kGy for aqueous frozen versus 6.29 kGy for aqueous) indicate that freezing slightly improves gamma radiation survival, and this improvement becomes more pronounced at higher radiation doses (**Sup. Fig. 1B, C**). All other gamma radiation survival curves shared a similar profile to the aqueous curve with no significant differences observed (**Fig. 11A, Sup. Fig. 1B, C**).

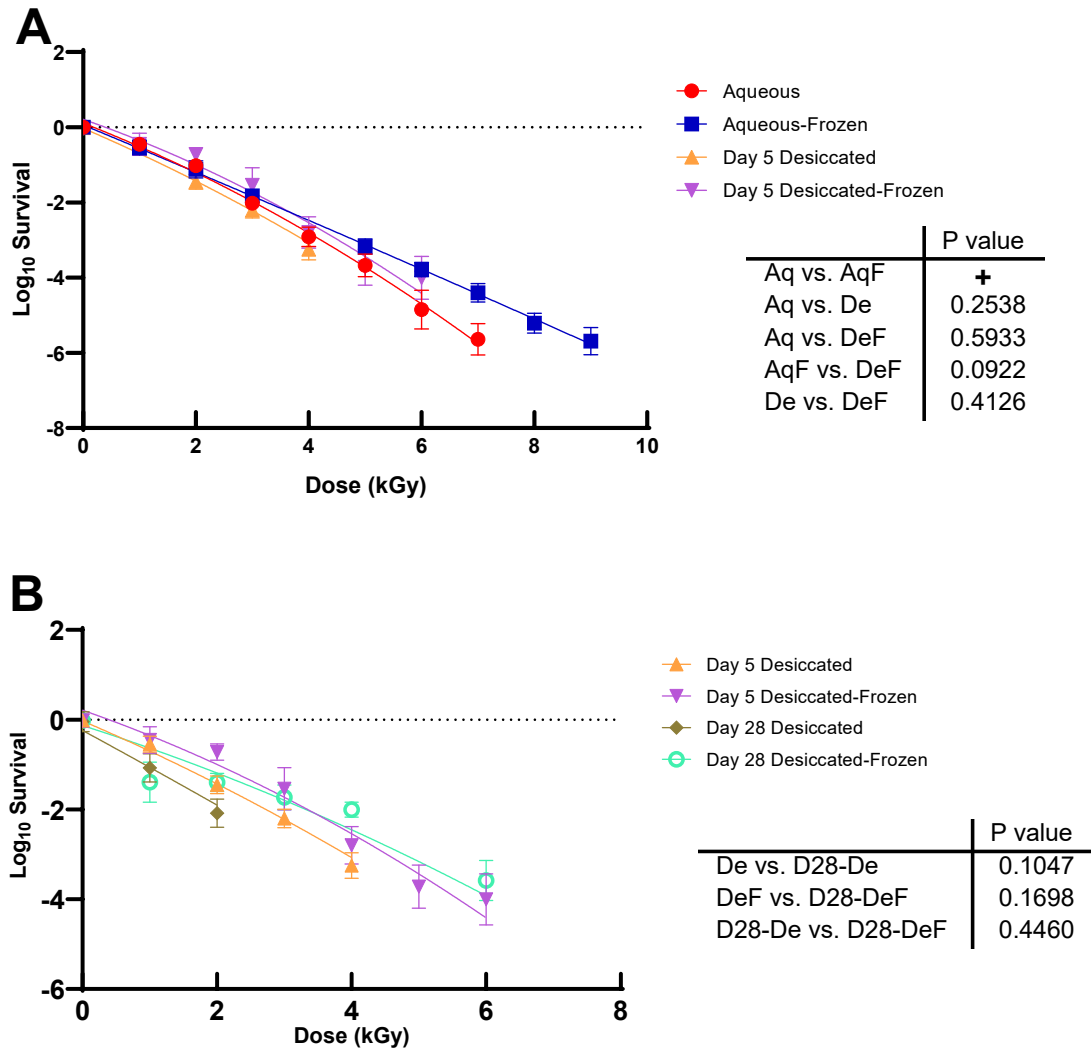


Figure 11. *B. subtilis* survival to gamma radiation under different physicochemical conditions. **A.** *B. subtilis* spore survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. subtilis* spore survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

For non-irradiated *B. subtilis* spores, freezing had no appreciable effect on survival, while 5-day desiccation had a 0.7-log decrease in survival. Freezing provided a slight improvement to desiccated survival. Extending desiccation to 28 days led to a pronounced drop in survival that was once again mitigated by freezing (Sup. Fig. 2).

B2-term constraint analysis for *B. subtilis* spores only prevented regression analysis of Day 28 desiccated data, with no other changes noted (**Sup. Fig. 21**). Aside from minor variations in curve fit and $D_{10}/D_{0.001}$ values for aqueous, aqueous-frozen, and Day 5 desiccated samples (**Sup. Fig. 5**), analysis of all sample data points did not alter the results in an appreciable manner (**Sup. Fig. 21**). Similarly, there were no major differences in non-irradiated survival values between the three analysis methods (**Sup. Fig. 2, 7, 8**).

CHAPTER 4: Discussion

Desiccation and ionizing radiation resistance have been associated with each other in the scientific literature for decades [70-74]; however, the exact nature and cause of that relationship has not been systematically studied. ROS formation has been associated with both forms of damage [20, 58, 72], and studies have pursued atmospheric exposure and metabolic disruption as possible sources of ROS in desiccation [70, 72, 76]. The key molecule associated with both forms of damage is water: its presence fuels radiolytic formation of ROS from ionizing radiation [20], while its absence exposes a cell to metabolic forms of ROS-mediated oxidative damage upon rehydration and recovery. Working through this dichotomy eventually led to the hypothesis tested and extended in this study:

“An environment that lessens the water content or changes the state of water in a cell (e.g. by drying and freezing), without killing the cell, should extend its radiation survival by limiting ROS formation.”

Desiccation improves gamma radiation resistance of most vegetative cells.

The key findings of this study are summarized in **Table 4**. In *E. coli*, *A. baumannii*, and *S. cerevisiae* EXF-6761, desiccation for five days significantly correlated to increased survival at both D_{10} and $D_{0.001}$ gamma radiation dose levels. The relative level of increase in these values was also higher than the increase noted from freezing alone (**Fig. 4, 5, 7**), and corroborate findings from previous DNA irradiation and food

Table 4: Summary of findings

Category	Species	Strain	Desiccation Improved Gamma Radiation Resistance?	Freezing Improved Gamma Radiation Resistance?	Desiccation/ Freezing Synergy?
Bacteria	<i>Escherichia coli</i>	K-12 (MG1655)	Yes	Yes	No
	<i>Acinetobacter baumannii</i>	AB5075	Yes	Yes	Yes
	<i>Lactobacillus plantarum</i>	ATCC 14917	No	Yes	N/A
Yeasts	<i>Saccharomyces cerevisiae</i>	EXF-6761	Yes	Yes	Yes
	<i>Saccharomyces cerevisiae</i>	EXF-6218	Yes	Yes	No
Bacterial Spores	<i>Bacillus megaterium</i>	QM B1551	No	No	No
	<i>Bacillus thuringiensis</i>	Al Hakam	No	No	No
	<i>Bacillus subtilis</i>	PS533	No	Yes	No

N/A: Not applicable

sterilization studies [32, 89, 92]. For *E. coli* and *A. baumannii*, this improved survival upon desiccation appeared to diminish slightly as radiation doses increased; for the yeast *S. cerevisiae* EXF-6761, the survival benefit increased at higher doses. Although there are too few observations to comment on correlation, they do indicate an avenue for further investigation with additional bacterial and eukaryotic strains.

L. plantarum demonstrated extreme desiccation sensitivity in this study (**Fig. 6, Sup. Fig. 2**), preventing analysis of desiccation effects on gamma radiation survival for this strain. *L. plantarum* is noted for not encoding any enzymatic antioxidants, instead relying on small metabolite-Mn(II) complexes for its ionizing radiation resistance [86, 121]. Additional Daly lab studies discovered that superoxide dismutase (SOD)-deficient mutant strains of *D. radiodurans* suffer a dramatic loss in desiccation resistance [122]. This possible link between SOD and desiccation resistance warrants further study with additional SOD-deficient strains to help clarify the linkage.

Freezing improves gamma radiation resistance of vegetative organisms.

While the primary focus of this study was the effects of desiccation on radiation resistance, deep freezing alone was also tested, both because of its previously-documented effects on DNA, protein, and contaminated food irradiation [32, 88-90], and because it is vital for studying how extreme environments can influence ionizing radiation resistance [93, 94]. For *E. coli*, *A. baumannii*, *L. plantarum*, and *S. cerevisiae* strains EXF-6761 and EXF-6218, freezing aqueous cultures at -80°C significantly improved their survival to gamma radiation (**Fig. 4-8**). Although the degree of benefit varied greatly between organisms, it was manifested at both low (D_{10}) and high ($D_{0.001}$)

doses. As mentioned in the Introduction, this benefit may likely stem from the ice lattice limiting ROS migration during irradiation. The fact that *B. megaterium* and *B. thuringiensis* spores did not display significantly improved survival in aqueous-frozen samples (**Fig. 9, 10**) is, most likely, the result of their greatly diminished water content (35-65%) [106] compared to vegetative cells. However, *B. subtilis* spores did show a significant survival benefit at higher doses from deep-freezing (**Fig. 11**).

Two important differences between *B. subtilis* spores and spores from *B. megaterium* and *B. thuringiensis* are that *B. subtilis* spores are monogenomic (one genome copy) and have a smaller spore size than *B. megaterium* and *B. thuringiensis*, which are digenomic (two genome copies) [105]. These differences suggest a benefit of freezing in radiation survival for certain spores based upon volume, but further testing is needed to validate this possibility.

Synergy between drying and freezing may relate to desiccation resistance.

In studying irradiated survival of Day 5 desiccated-frozen samples, two vegetative organisms had a cumulative gamma radiation survival benefit between freezing and desiccation: *A. baumannii* and *S. cerevisiae* EXF-6761 (**Fig. 5, 7**). *E. coli* and *S. cerevisiae* EXF-6218, however, did not demonstrate this synergistic effect. Looking closer, these organisms shared another trait: *A. baumannii* and EXF-6761 gave lower population losses from desiccation alone (0.46 and -0.65 logs, respectively) when compared to *E. coli* and EXF-6218 (-1.64 and -1.07 logs, respectively) (**Sup. Fig. 2**).

These findings support a correlation between desiccation resistance and the synergistic benefits of desiccation and freezing on gamma radiation survival. One

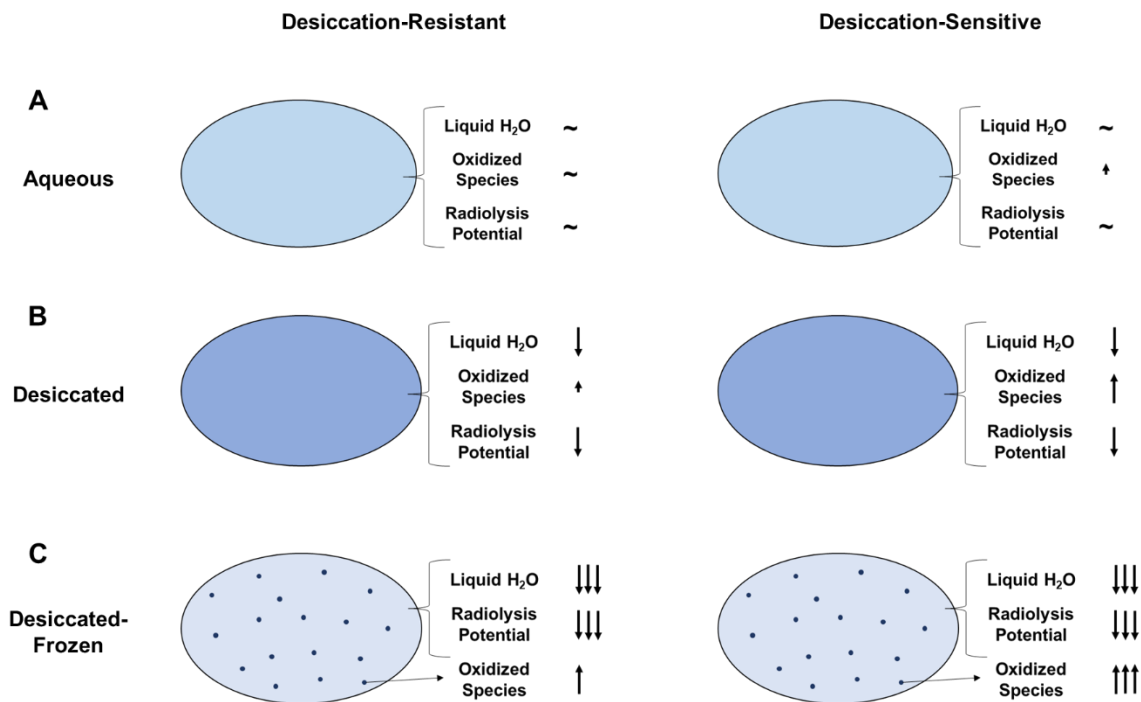


Figure 12. Model of relationship between desiccation resistance and desiccation-freezing synergy in gamma radiation survival. **A.** Model of two cells, one desiccation-resistant, one desiccation-sensitive, under aqueous conditions; and relative levels of water, potential for ionizing radiation radiolysis, and oxidative species (oxidized organic radicals and long-lived ROS). **B.** Model of two desiccated cells, one desiccation-resistant, one desiccation-sensitive; and relative levels of water, potential for ionizing radiation radiolysis, and oxidative species (oxidized organic radicals and long-lived ROS). **C.** Model of two desiccated cells, one desiccation-resistant, one desiccation-sensitive, that have undergone freezing at -80°C and excluded cytosolic solutes to small concentrated spaces between ice crystals (dots); and the cell's relative levels of water, potential for ionizing radiation radiolysis, and oxidative species (oxidized organic radicals and long-lived ROS) within the excluded spaces. ~: Normal/unaltered levels. Cell components and arrows not drawn to scale.

explanation for this trend is that when organic solutions freeze, solutes are excluded from the solvent by the formation of ice crystals, concentrating in small spaces throughout the ice lattice [123]. In desiccation-resistant organisms, ROS buildup is inhibited by Mn(II) antioxidants [70-73], resulting in fewer oxidative species (ROS and oxidized macromolecular radicals (proteins, lipids, nucleic acids, etc.)) accumulated in regions between ice crystals. However, desiccation-sensitive organisms accumulate higher concentrations of oxidized species, which proliferate their damage to other organic macromolecules. This could prime the organism for ionizing radiation injury, negating

any radioprotection that ice provides (**Fig. 12**). Researchers have observed protein oxidation occurring when cells are frozen at -80°C [124]. This hypothesis could be helpful in predicting the extent of synergy between desiccation and freezing on an organism's ionizing radiation resistance. However, further study on additional desiccation-resistant and -sensitive strains is required, potentially using fluorescent probes for oxidative species (see Abo, *et al.* [125] for an example) to monitor relative locations and concentrations of these species in desiccated and desiccated-frozen cells.

Desiccating bacterial spores did not enhance their gamma radiation survival.

For *Bacillus* spore strains, there was no observed desiccation-induced benefit to gamma radiation survival. With *B. subtilis*, the Day 5 desiccated and desiccated-frozen survival curves were nearly identical to the aqueous curve (**Fig. 11**). This was anticipated from the limited intracellular water in the spore [106], suggesting that changes in water levels would not be as pronounced in spores compared to vegetative cells. This would result in less change in ROS production for desiccated spores versus desiccated vegetative cells and less overall survival benefit from desiccation.

However, *B. megaterium* and *B. thuringiensis* shared an interesting trend regarding desiccation and gamma radiation survival. Both appeared to have a decrease in survival with Day 5 desiccated versus aqueous samples (**Fig. 9, 10**), although they were not statistically significant. Additional studies with more data points in the observed gamma radiation survival ranges for Day 5 desiccated samples could improve the ability to statistically analyze differences between these curves and their aqueous counterparts.

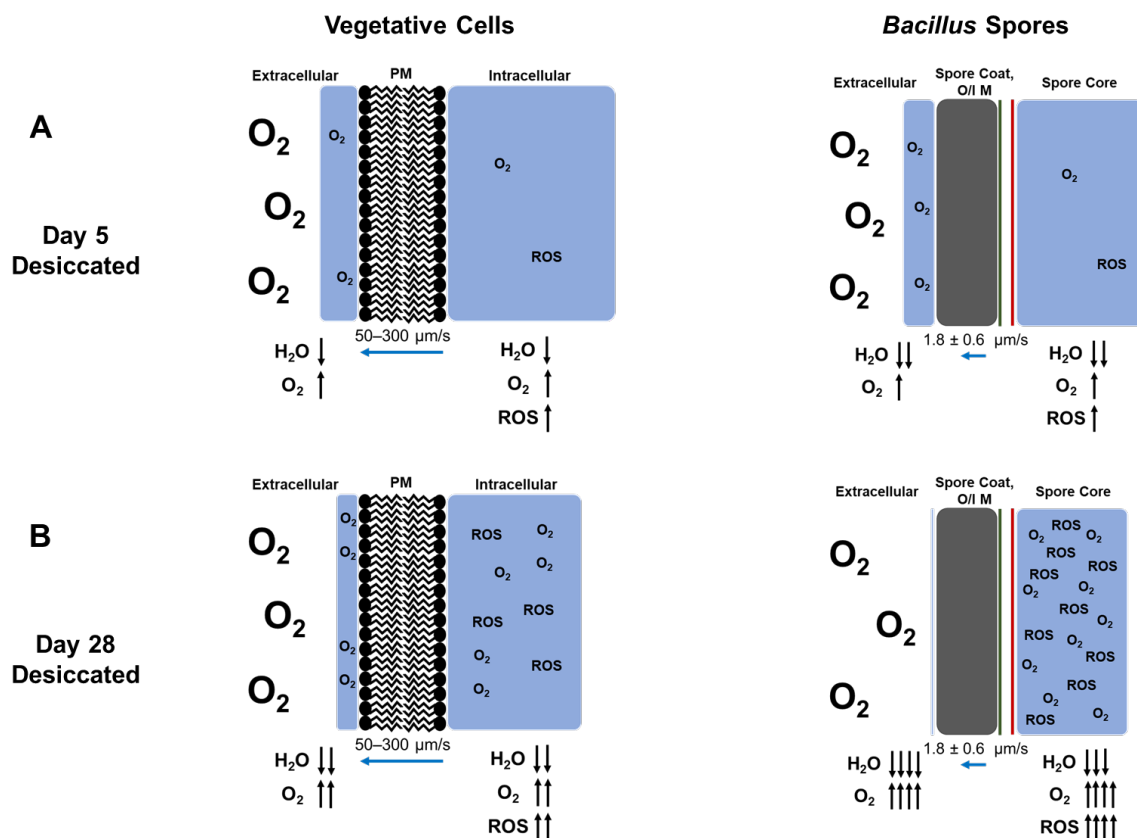


Figure 13. Model of desiccation duration effects on vegetative cells versus *Bacillus* spores. **A.** Model of a vegetative cell and a *Bacillus* spore after 5 days of desiccation. **B.** Model of a vegetative cell and a *Bacillus* spore after 28 days of desiccation. Blue fields represent relative water content in extracellular versus intracellular/spore core spaces. Blue arrows indicate direction and relative rate of water diffusion across either plasma membrane (PM) or spore covering (spore coat and outer/inner membranes (O/I M)), with literature values for both provided [126-129]. Black arrows represent relative levels of water, oxygen, and ROS. Cell components and arrows not drawn to scale.

The *Bacillus* spores also had much greater non-irradiated survival decreases compared to the vegetative strains, especially for Day 28 desiccated samples (**Sup. Fig. 2**). With no active metabolism occurring in the spores [130, 131], the desiccation cell damage most likely comes from the dried spores being exposed to atmospheric oxygen [70, 72, 76]. Water cannot pass through a spore coat and membranes nearly as fast as a plasma membrane, and spores have little intracellular water reserves, which would mean water evaporating from the surface of a spore cannot be replaced as easily as it would for a vegetative cell [106, 126-129]. Without a water barrier, atmospheric oxygen has

greater access and could cause significant damage to spores, especially larger spores like *B. megaterium* and *B. thuringiensis* with their greater exposed surface area (**Fig. 13**).

Further studies measuring the external and internal water content of *Bacillus* spores of different sizes over different periods of desiccation are called for to pursue this idea.

Desiccation: Selective pressure or less radiolysis?

Desiccation's effects on gamma radiation survival could be explained by desiccation itself placing selective pressure on organism cultures, favoring resistant organism survival that leads to improved relative gamma radiation resistance. With the link between desiccation and ionizing radiation resistance [70-76], one could suspect that organisms that survive desiccation would be better equipped to survive gamma radiation.

Several steps used in this study helped to control for possible genetic variation in the strains used. The strains chosen are established and well-maintained cell lines that have been studied extensively and are well documented (**Table 1**), reducing the likelihood of variance within each strain. Also, single colonies were used to seed cultures, promoting uniformity within each culture to reduce genotype or phenotype variation.

Multiple study findings also favor reduced water radiolysis over selective pressure as the cause of increased gamma radiation resistance from desiccation. The degree of resistance increase noted (up to an 8.5-fold increase for *A. baumannii*) favors a more drastic alteration than minor changes to an organism's genome (**Fig. 4, 5, 7, 8**). Multiple vegetative organisms experienced a similar survival increase from desiccation, making it unlikely that genetic variance is the cause of the increase. Finally, *Bacillus* spores, with

their reduced water content, did not experience a similar increase in gamma radiation resistance when desiccated (**Fig. 9-11**), favoring the reduced water radiolysis hypothesis over the selective pressure hypothesis.

Practical implications of these findings.

Sterilization techniques. The medical and food industries have studied the impacts of freezing and drying on ionizing radiation resistance during irradiation sterilization [6, 89, 91, 92]. However, as this thesis research demonstrates, the impacts vary greatly between different types of organisms. These variations must be considered when designing ionizing radiation sterilization protocols. For example, irradiating food or equipment under extremely dry conditions could reduce bacterial spore survival, while irradiating under humid or hydrated conditions could reduce vegetative organism survival.

Biological defense. The threat of biological agents (such as *Bacillus anthracis*) used against military and civilian populations remains a concern for military and homeland defense planning [132]. The “anthrax letters” of 2001 are a prime example of how biological agents can be delivered in multiple different ways and how effective ionizing radiation is at decontaminating these agents [132, 133]. The findings from this study, though, reveal that the way a biological agent is prepared and stored could greatly enhance its resistance to ionizing radiation, making it critical to know the environmental conditions (temperature, moisture, etc.) of a container and its contents prior to radiation sterilization to ensure a high enough dose is used to be effective.

Space exploration. Researchers have spent decades trying to determine how the environment of space and space travel can affect living organisms [93]. Although this environment is extremely hostile (cold, dry vacuum; constant exposure to cosmic radiation), previous research [94] and this study show that some of these conditions (cold, dry) greatly enhance radiation survival and could promote the survival of desiccation-resistant organisms in space. This raises the concern that space exploration missions could unintentionally transfer living microorganisms from other planets back to Earth, and *vice versa*.

Future studies.

This research suggests several additional avenues of study that could expand upon and clarify the significant findings, trends, and hypotheses discussed here. The most important additional experiment set needed to accompany these findings is measuring the internal and external water content of aqueous and desiccated cells to determine how much water loss takes place during desiccation. Several highly-technical methods, such as time-domain reflectometry [134, 135] and suspended microchannel resonators with H₂O / D₂O water exchange [128] have been used to measure water content inside spores and cells; however, these methods require specialized equipment and are useful only for cells in aqueous solutions, not desiccated samples. Older and more standardized methods, such as oven drying, have their own technical issues and cannot distinguish between extracellular and intracellular water [136]. More study is required to find a cellular water measuring protocol that can specifically distinguish residual extracellular and intracellular water levels in desiccated samples.

Another key piece of information from the studied samples would be data on the levels of DNA DSBs and protein carbonylation that occurs during sample irradiation under desiccated or frozen conditions. This would help determine whether these physicochemical conditions influence DNA or protein damage more to promote increased gamma radiation resistance in vegetative organisms. Pulse field gel electrophoresis (PFGE), which is used to identify large DNA fragments by length [137], has been used by several labs to measure DSB yields from irradiation [49, 80]. Protein carbonyl assays, which measure CO-group presence on proteins, is the current standard for measuring oxidative damage to proteins [138]. These two methods could be used on lysates from irradiated cells to measure their levels of DNA and protein damage relative to non-irradiated cells, giving insight into which type of damage is most reduced by desiccation or freezing.

While water content, DNA damage, and protein damage measurements would add depth to the findings of this study, including different types of organisms, would greatly increase the study's breadth. One such group of organisms would be the germinated cell forms of the studied *Bacillus* strains. In their germinated state, *Bacillus* strains are very similar to other vegetative organisms in form and function [135]. Therefore, one would expect to see significant improvements in gamma radiation survival in aqueous-frozen versus aqueous samples for all three studied strains in their germinated state. As for their desiccated state, increases in gamma radiation survival would be expected, as well. However, as was seen with *S. cerevisiae* EXF-6218 (**Fig. 8**), there may be potential challenges to accurate regression modeling if the act of desiccation triggers the *Bacillus* strains to sporulate again.

Another “organism” to test under the various physicochemical states would be viruses, both in extracellular and intracellular conditions. Virus particles on their own consist of protein coats that encapsulate small, tightly packed DNA or RNA genomes. Viral radiation survival is traditionally quantified *in vitro* for purified preparations held in isotonic buffer. This study’s findings, though, indicate that viruses maintained within cellular hosts during irradiation would be much more radiation resistant because of the host’s ROS defenses; and this resistance would be enhanced under desiccated or frozen conditions [139]. These hypotheses, however, remain unexplored.

Lyophilization, also known as freeze-drying, provides another way to expand the study’s breadth. The act of lyophilization creates a new type of physicochemical state, where water is first frozen, then sublimated from a sample via a reduction in pressure. This process can reduce a cell’s water content much faster than desiccation and with less stress [140]. Because a cell would not experience nearly the same degree of ROS-induced oxidative damage as it would during desiccation, while maintaining the same degree of water loss, its gamma radiation survival could be significantly higher in a lyophilized state versus a desiccated or desiccated-frozen state. This increase could also extend to desiccation-sensitive organisms, such as *L. plantarum*. Indeed, lyophilization, with its reduced pressures, low temperatures, and dry conditions, could even serve as a better analog for space conditions [93], thereby providing improved estimates on potential cosmic radiation resistance for a variety of microorganisms.

Conclusion.

Our knowledge of ionizing radiation is always changing, leading to new discoveries and new understandings. Even knowing the importance of water to ionizing radiation injury in organisms, the variety and degree of survival response to either its removal (drying) or state change (freezing) reveal new insights into the nature of desiccation injury and ice formation in biological systems. The implications for commercial radiation sterilization, biological defense, and space exploration are also crucial, encouraging a reevaluation of the procedures currently used in these fields to improve efficacy and safety. Desiccation and deep freezing improve gamma radiation resistance in many vegetative organisms that survive these processes, but the greater findings lie in the unique microorganism responses, paving the way to new and exciting avenues of study and a greater understanding and appreciation for the utility and influence of ionizing radiation.

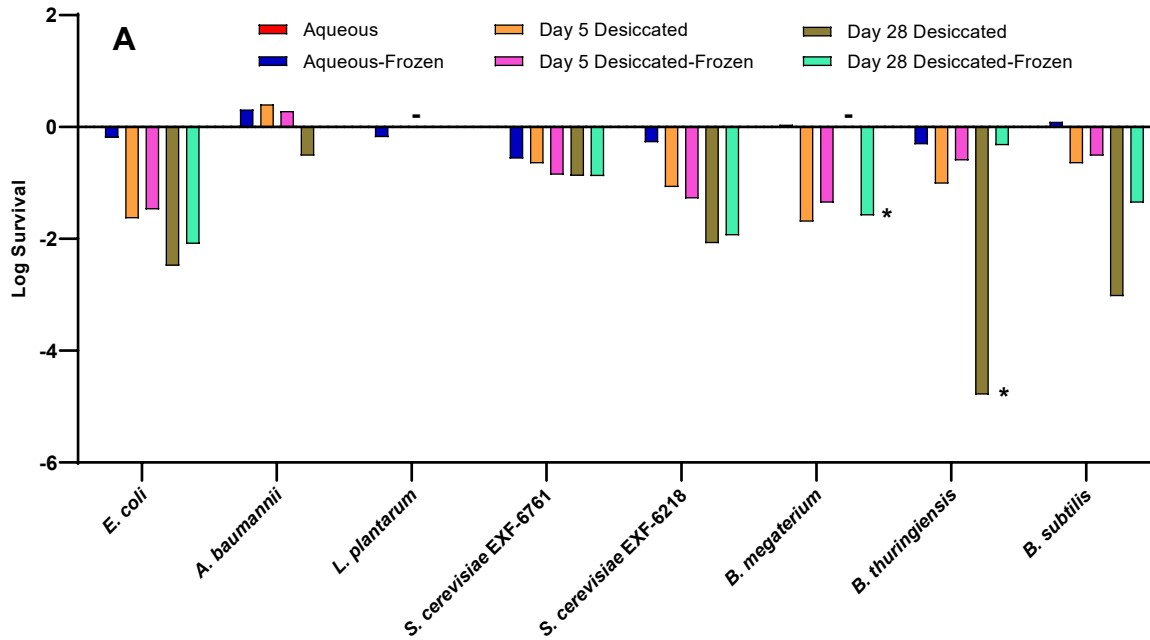
Supplemental Figures

A	Adj. R ²						
		Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		0.998	0.993	0.987	0.988	0.990	0.994
<i>A. baumannii</i>		0.973	0.992	0.986	0.977	0.988	0.986
<i>L. plantarum</i>		1.000	0.999	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		0.993	0.964	0.989	0.960	0.896	0.998
<i>S. cerevisiae</i> EXF-6218		0.941	0.978	0.974	0.908	0.690	0.982
<i>B. megaterium</i>		0.974	0.963	1.000	1.000	X	X
<i>B. thuringiensis</i>		0.962	0.972	1.000	1.000	X	0.977
<i>B. subtilis</i>		0.995	0.999	0.996	0.956	1.000	0.854

B	D ₁₀ (kGy)						
		Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		0.52	0.69	1.45	0.87	1.54	2.05
<i>A. baumannii</i>		0.14	0.34	1.19	1.49	1.09	0.93
<i>L. plantarum</i>		0.79	3.78	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		3.68	7.06	7.38	7.94	5.39	5.25
<i>S. cerevisiae</i> EXF-6218		1.71	3.27	2.03	1.21	5.90	1.72
<i>B. megaterium</i>		2.56	2.79	1.57	2.85	X	X
<i>B. thuringiensis</i>		2.51	2.32	1.48	2.03	X	2.35
<i>B. subtilis</i>		1.71	1.69	1.37	1.93	0.87	1.34

C	D _{0.001} (kGy)						
		Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		2.82	4.02	7.41	6.84	7.52	7.87
<i>A. baumannii</i>		0.84	1.57	4.91	6.27	5.14	4.94
<i>L. plantarum</i>		3.03	11.76	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		8.15	18.24	21.14	26.96	21.97	21.88
<i>S. cerevisiae</i> EXF-6218		4.54	12.46	#	#	#	#
<i>B. megaterium</i>		9.28	9.43	5.46	8.61	X	X
<i>B. thuringiensis</i>		9.83	9.67	4.85	7.68	X	7.51
<i>B. subtilis</i>		6.29	7.85	6.31	6.73	11.10	8.61

Supplemental Figure 1. Regression fit and derived D₁₀ and D_{0.001} values for all organisms. A. Adjusted R² values for survival curves of the six physicochemical conditions: aqueous, aqueous-frozen, Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, Day 28 desiccated-frozen. **B.** D₁₀ values (in kGy) derived from each survival curve. **C.** D_{0.001} values (in kGy) derived from each survival curve. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: too few survivors to analyze condition; x: too few data points to model survival curve; #: survival curve unable to reach value due to positive B2 term.



B

	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>	0.000	-0.195	-1.636	-1.475	-2.482	-2.090
<i>A. baumannii</i>	0.000	0.313	0.407	0.284	-0.514	0.007
<i>L. plantarum</i>	0.000	-0.180	-	-	-	-
<i>S. cerevisiae</i> EXF-6761	0.000	-0.567	-0.651	-0.854	-0.872	-0.879
<i>S. cerevisiae</i> EXF-6218	0.000	-0.275	-1.073	-1.277	-2.080	-1.940
<i>B. megaterium</i>	0.000	0.042	-1.693	-1.353	-	-1.582*
<i>B. thuringiensis</i>	0.000	-0.310	-1.015	-0.599	-4.787*	-0.327
<i>B. subtilis</i>	0.000	0.095	-0.652	-0.513	-3.026	-1.354

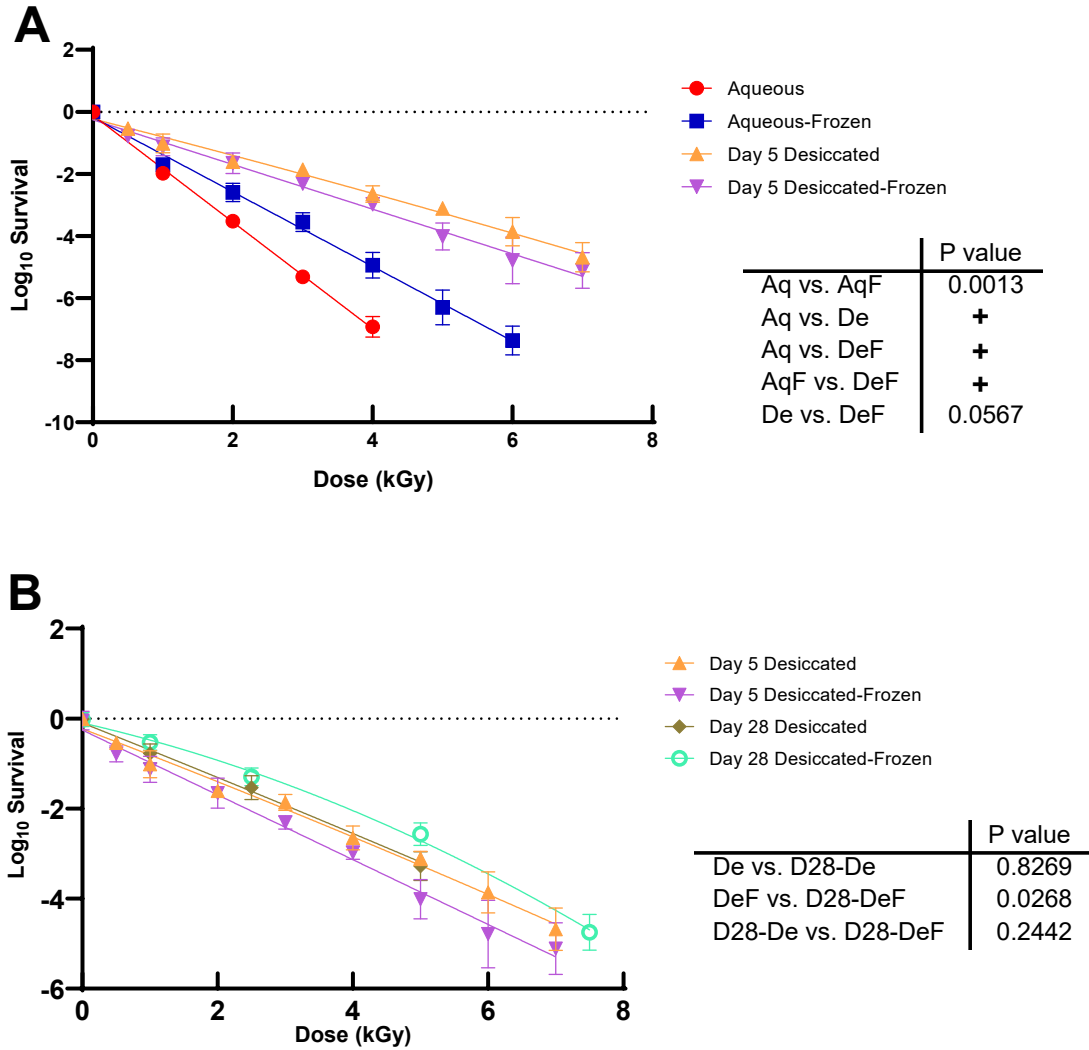
Supplemental Figure 2. Organism population losses independent of gamma radiation under different physicochemical conditions. Non-irradiated log survival readings were derived from each survival curve for each condition (aqueous, aqueous-frozen, Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, Day 28 desiccated-frozen) and microorganism studied. Readings were then normalized with aqueous sample baseline removed. **A.** Graphical representation of non-irradiated survival data. **B.** Actual values of non-irradiated survival data. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: no survivors at any dose for this cohort; *: no regression curve available, measured non-irradiated value used.

A	Adj. R²	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		0.997	0.993	0.987	0.988	0.990	0.994
<i>A. baumannii</i>		0.754	0.974	0.986	0.977	0.981	0.985
<i>L. plantarum</i>		1.000	0.999	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		0.993	0.964	0.989	0.960	0.896	0.998
<i>S. cerevisiae</i> EXF-6218		0.941	0.978	0.100	0.863	0.690	0.946
<i>B. megaterium</i>		0.974	0.963	1.000	1.000	X	X
<i>B. thuringiensis</i>		0.962	0.972	1.000	1.000	X	0.977
<i>B. subtilis</i>		0.995	0.999	0.996	0.956	X	0.854

B	D₁₀ (kGy)	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		0.52	0.69	1.45	0.87	1.54	2.05
<i>A. baumannii</i>		-0.09	0.40	1.19	1.49	1.10	0.92
<i>L. plantarum</i>		0.79	3.78	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		3.68	7.06	7.38	7.94	5.39	5.25
<i>S. cerevisiae</i> EXF-6218		1.71	3.27	2.62	1.19	6.02	1.89
<i>B. megaterium</i>		2.56	2.79	1.57	2.85	X	X
<i>B. thuringiensis</i>		2.51	2.32	1.48	2.03	X	2.35
<i>B. subtilis</i>		1.71	1.69	1.37	1.93	X	1.34

C	D_{0.001} (kGy)	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		2.84	4.02	7.41	6.84	7.52	7.87
<i>A. baumannii</i>		1.01	1.50	4.91	6.27	5.12	4.95
<i>L. plantarum</i>		3.03	11.76	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		8.15	18.24	21.14	26.96	21.97	21.88
<i>S. cerevisiae</i> EXF-6218		4.54	12.46	13.22	11.80	19.50	15.37
<i>B. megaterium</i>		9.28	9.43	5.46	8.61	X	X
<i>B. thuringiensis</i>		9.83	9.67	4.85	7.68	X	7.51
<i>B. subtilis</i>		6.29	7.85	6.31	6.73	X	8.61

Supplemental Figure 3. Regression fit and derived D₁₀ and D_{0.001} values for all organisms, B2 regression coefficients constrained to negative values. **A.** Adjusted R² values for survival curves of the six physicochemical conditions: aqueous, aqueous-frozen, Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, Day 28 desiccated-frozen. **B.** D₁₀ values (in kGy) derived from each survival curve. **C.** D_{0.001} values (in kGy) derived from each survival curve. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: too few survivors to analyze condition; x: too few data points to model survival curve.



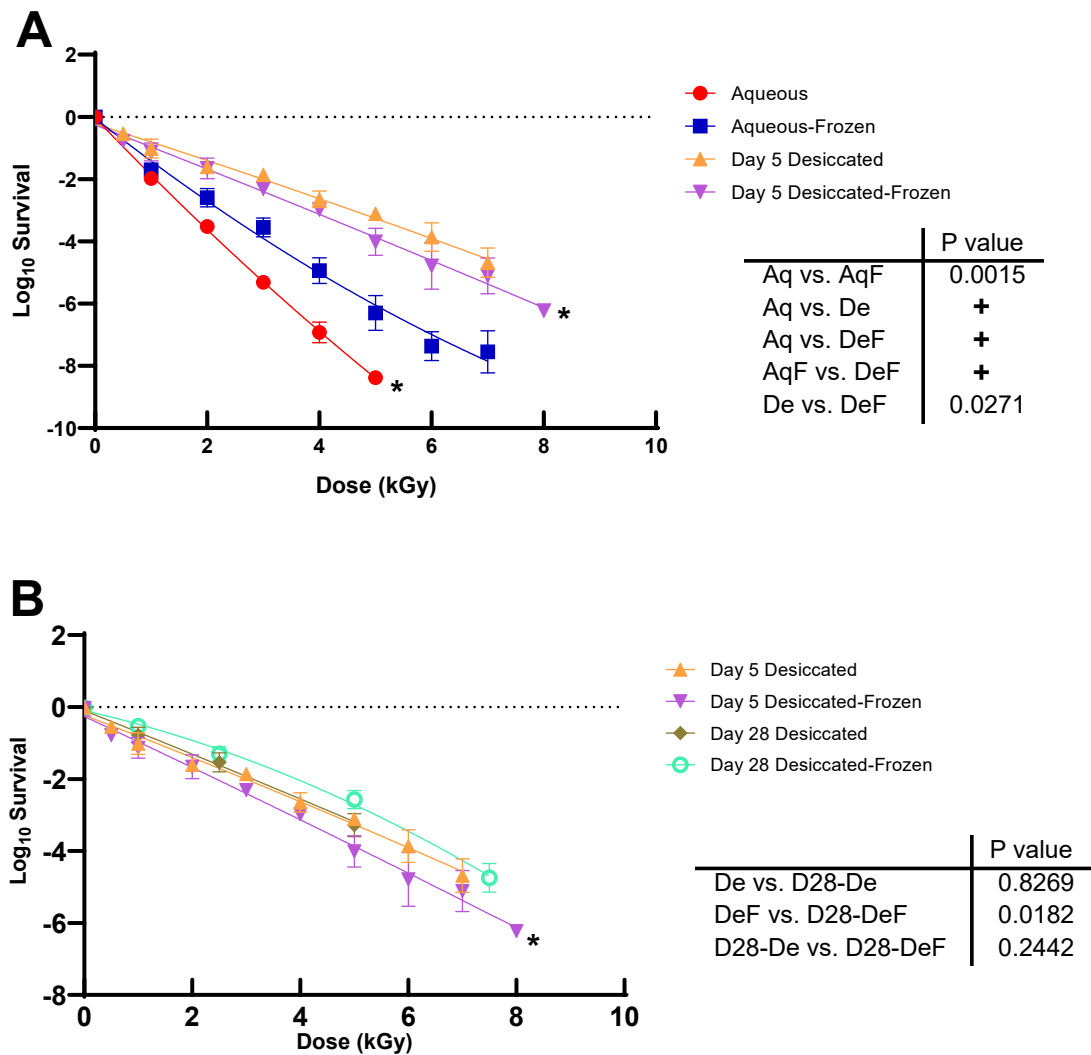
Supplemental Figure 4. *E. coli* survival to gamma radiation under different physicochemical conditions; B2 regression coefficients constrained to negative values. A. *E. coli* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *E. coli* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.

A	Adj. R²	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		0.999	0.986	0.987	0.991	0.990	0.994
<i>A. baumannii</i>		0.973	0.984	0.983	0.979	0.980	0.981
<i>L. plantarum</i>		1.000	0.999	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		0.990	0.976	0.989	0.960	0.951	0.997
<i>S. cerevisiae</i> EXF-6218		0.973	0.978	0.983	0.978	0.690	0.969
<i>B. megaterium</i>		0.987	0.965	1.000	0.883	X	X
<i>B. thuringiensis</i>		0.977	0.974	1.000	0.994	X	0.974
<i>B. subtilis</i>		0.976	0.997	0.978	0.956	1.000	0.854

B	D₁₀ (kGy)	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		0.52	0.69	1.32	1.05	1.54	2.05
<i>A. baumannii</i>		0.04	0.35	1.17	1.45	1.07	0.90
<i>L. plantarum</i>		0.79	3.78	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		3.51	7.38	7.38	7.94	5.24	5.76
<i>S. cerevisiae</i> EXF-6218		1.62	3.27	2.17	1.26	6.70	1.70
<i>B. megaterium</i>		2.33	2.67	1.13	2.21	X	X
<i>B. thuringiensis</i>		2.36	2.10	1.48	1.87	X	1.80
<i>B. subtilis</i>		1.53	1.65	1.37	1.81	0.87	1.34

C	D_{0.001} (kGy)	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>		2.82	3.98	7.65	6.51	7.52	7.87
<i>A. baumannii</i>		0.94	1.45	4.93	6.49	5.27	4.96
<i>L. plantarum</i>		3.03	11.76	-	-	-	-
<i>S. cerevisiae</i> EXF-6761		8.30	17.54	21.14	26.96	20.63	20.91
<i>S. cerevisiae</i> EXF-6218		4.72	12.46	#	#	#	#
<i>B. megaterium</i>		9.91	10.20	7.91	9.26	X	X
<i>B. thuringiensis</i>		10.52	10.18	4.85	8.38	X	9.33
<i>B. subtilis</i>		6.77	7.98	6.69	7.09	11.10	8.61

Supplemental Figure 5. Regression fit and derived D₁₀ and D_{0.001} values for all organisms; all data points analyzed. **A.** Adjusted R² values for survival curves of the six physicochemical conditions: aqueous, aqueous-frozen, Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, Day 28 desiccated-frozen. **B.** D₁₀ values (in kGy) derived from each survival curve. **C.** D_{0.001} values (in kGy) derived from each survival curve. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: too few survivors to analyze condition; x: too few data points to model survival curve; #: survival curve unable to reach value due to positive B2 term.



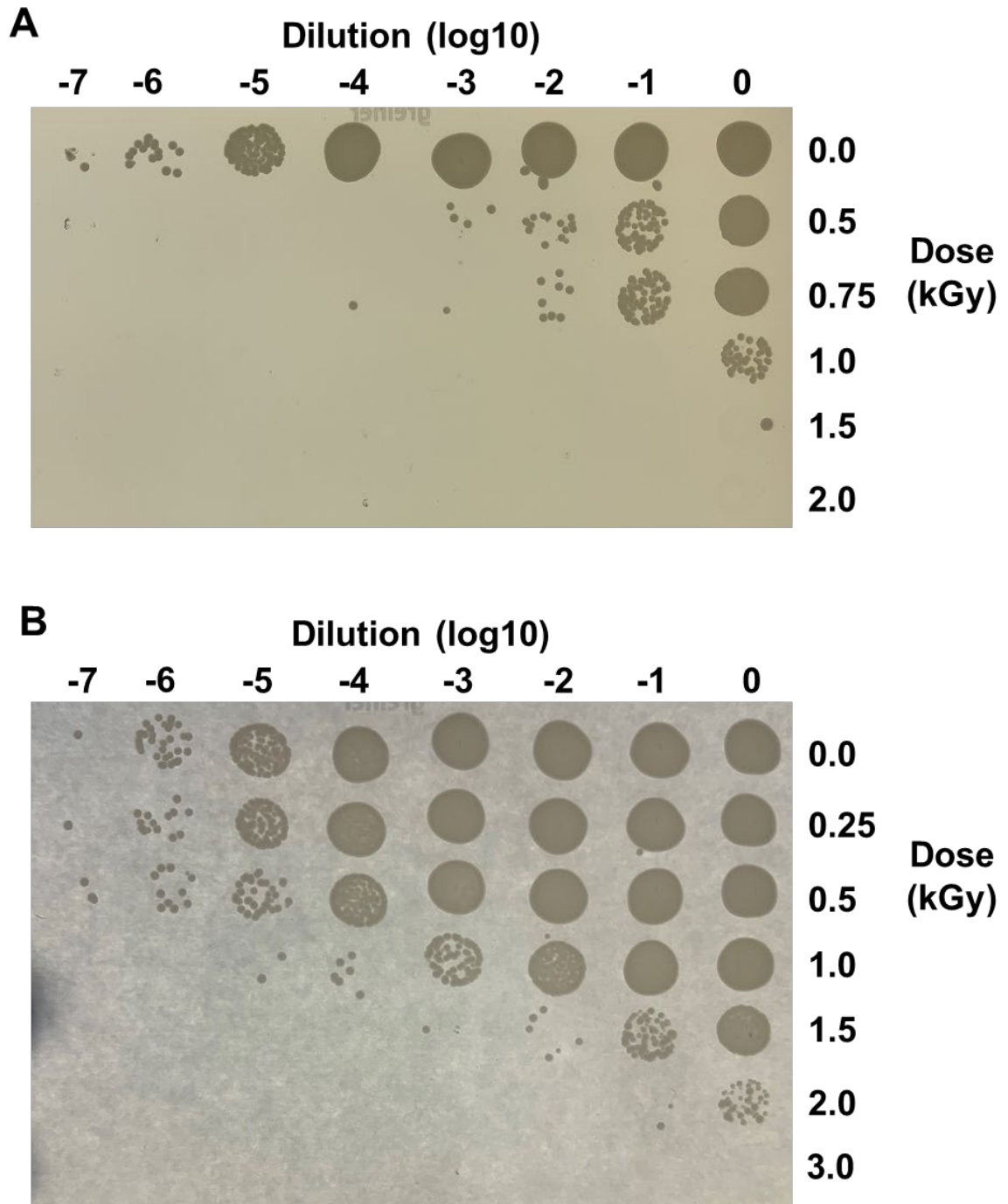
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	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>	0.000	-0.127	-1.568	-1.444	-2.415	-2.023
<i>A. baumannii</i>	0.000	0.337	0.373	0.308	-0.691	-0.042
<i>L. plantarum</i>	0.000	-0.180	-	-	-	-
<i>S. cerevisiae</i>EXF-6761	0.000	-0.567	-0.651	-0.854	-0.872	-0.879
<i>S. cerevisiae</i>EXF-6218	0.000	-0.275	-1.073	-1.277	-2.080	-1.940
<i>B. megaterium</i>	0.000	0.042	-1.693	-1.353	-	-1.582 *
<i>B. thuringiensis</i>	0.000	-0.310	-1.015	-0.599	-4.787 *	-0.327
<i>B. subtilis</i>	0.000	0.095	-0.652	-0.513	-3.026	-1.354

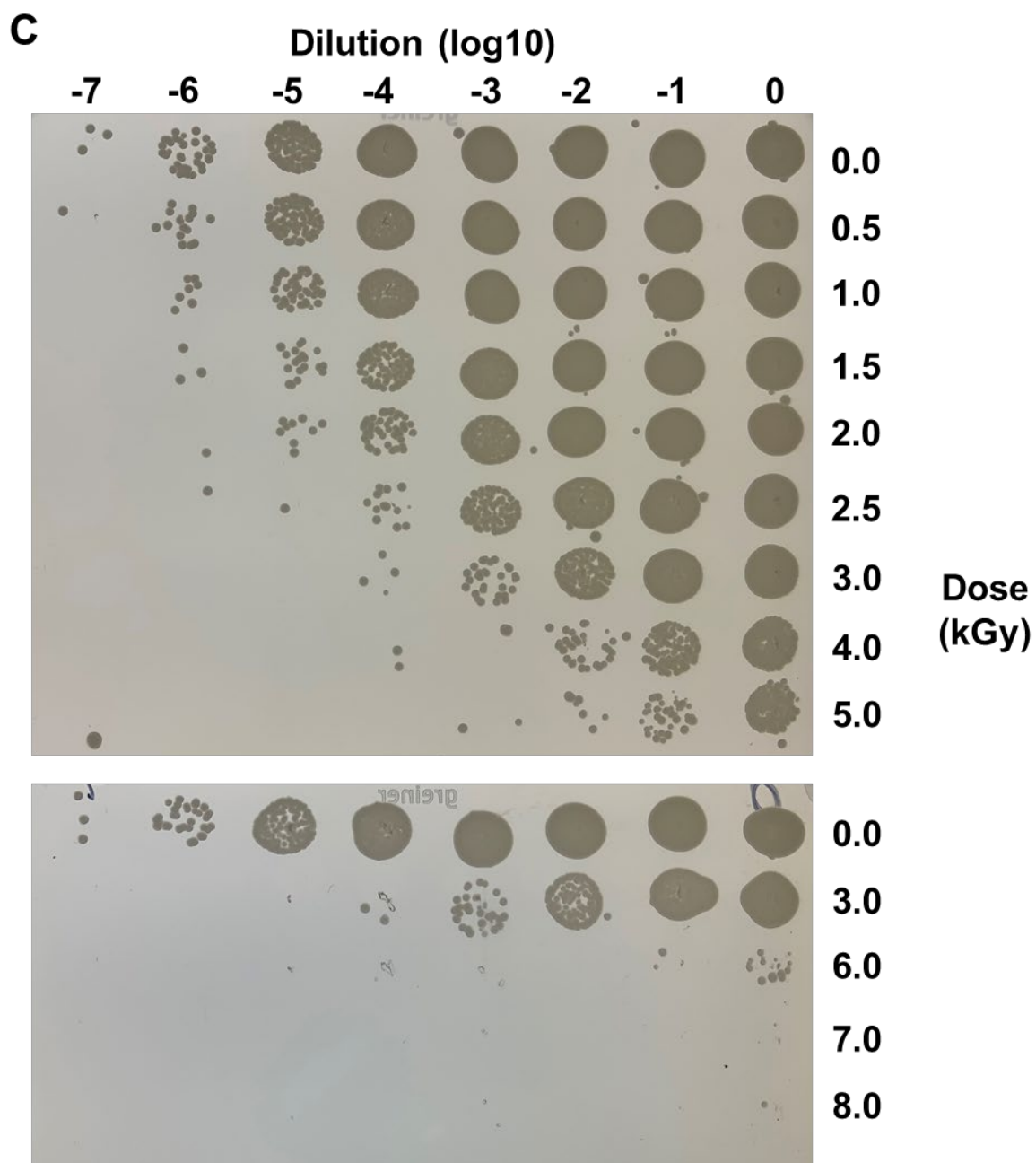
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	Aq	AqF	De	DeF	D28-De	D28-DeF
<i>E. coli</i>	0.000	-0.081	-1.645	-1.504	-2.491	-2.099
<i>A. baumannii</i>	0.000	0.535	0.543	0.379	-0.403	0.193
<i>L. plantarum</i>	0.000	-0.180	-	-	-	-
<i>S. cerevisiae</i>EXF-6761	0.000	-0.682	-0.733	-0.936	-1.006	-0.998
<i>S. cerevisiae</i>EXF-6218	0.000	-0.342	-1.144	-1.377	-2.146	-2.000
<i>B. megaterium</i>	0.000	0.108	-1.721	-1.176	-	-1.610 *
<i>B. thuringiensis</i>	0.000	-0.341	-1.151	-0.678	-4.923 *	-0.381
<i>B. subtilis</i>	0.000	-0.118	-0.811	-0.781	-3.294	-1.622

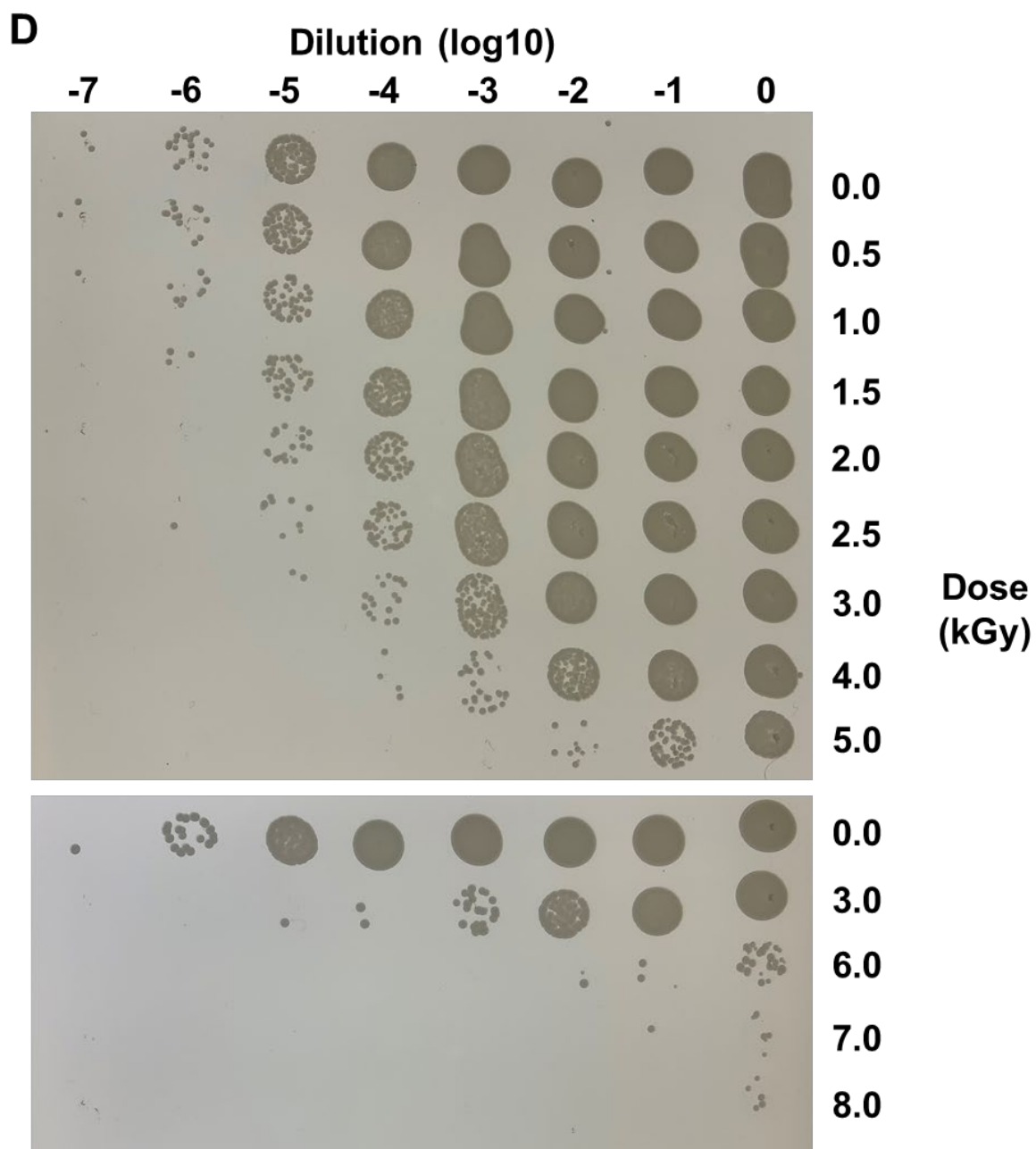
Supplemental Figure 8. Organism population losses independent of gamma radiation under different physicochemical conditions; all data points analyzed. Non-irradiated log survival readings were derived from each survival curve for each condition (aqueous, aqueous-frozen, Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, Day 28 desiccated-frozen) and microorganism studied. Readings were then normalized with aqueous sample baseline removed. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: no survivors at any dose for this cohort; *: no regression curve available, measured non-irradiated value used.



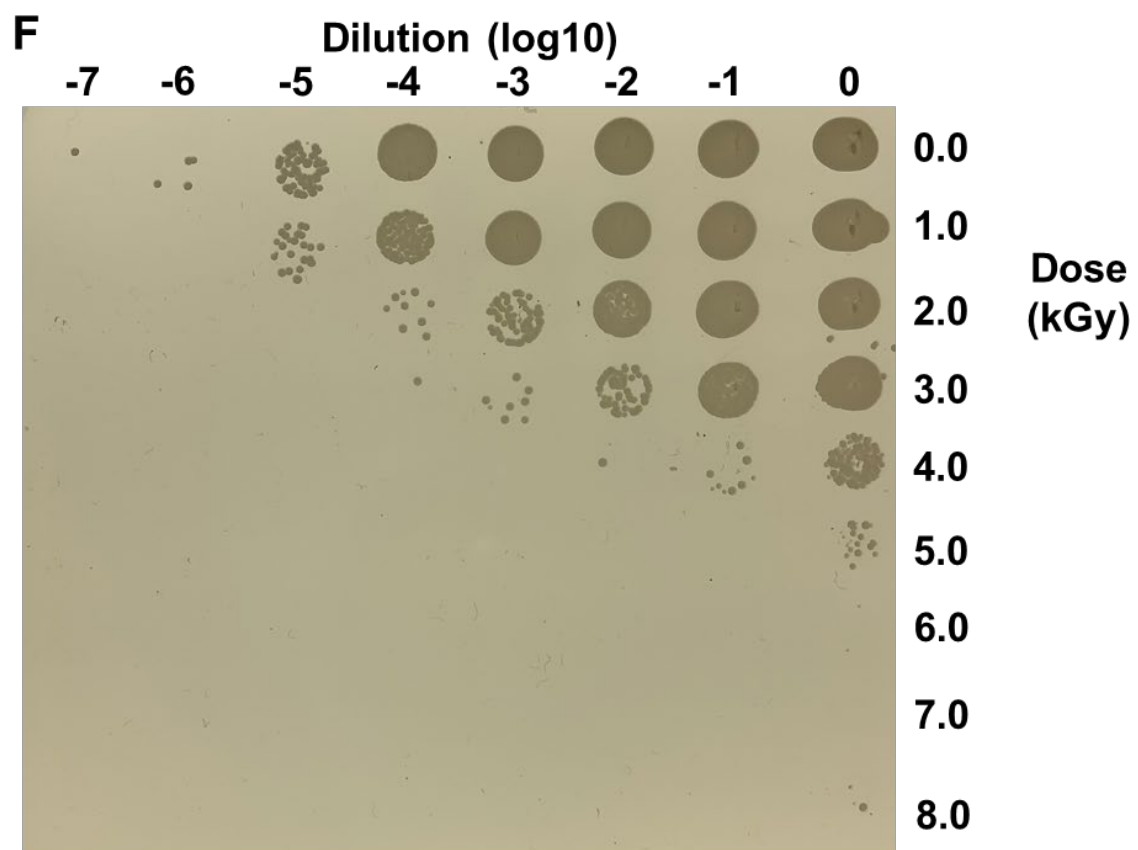
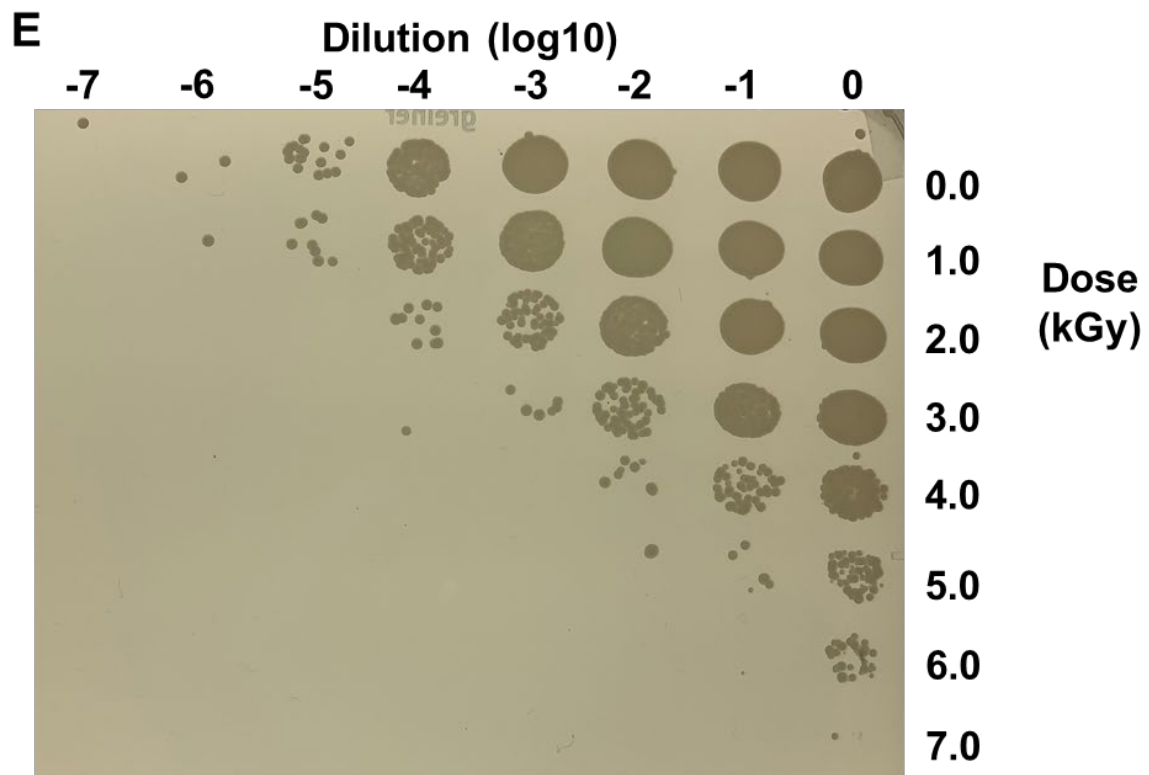
Supplemental Figure 9. *A. baumannii* spot dilution survival assays. Photographs taken of each dilution assay plate for each cohort. Photographs are representative of two replicates. **A.** Aqueous. **B.** Aqueous-frozen. **C.** Day 5 (upper) and Day 6 (lower) desiccated. **D.** Day 5 (upper) and Day 6 (lower) desiccated-frozen. **E.** Day 28 desiccated. **F.** Day 28 desiccated-frozen.



Supplemental Figure 9. *A. baumannii* spot dilution assays (continued).



Supplemental Figure 9. *A. baumannii* spot dilution assays (continued).



Supplemental Figure 9. *A. baumannii* spot dilution assays (continued).

Assuming Day 6 survival values proportional to Day 5 survival values at same dose, or:

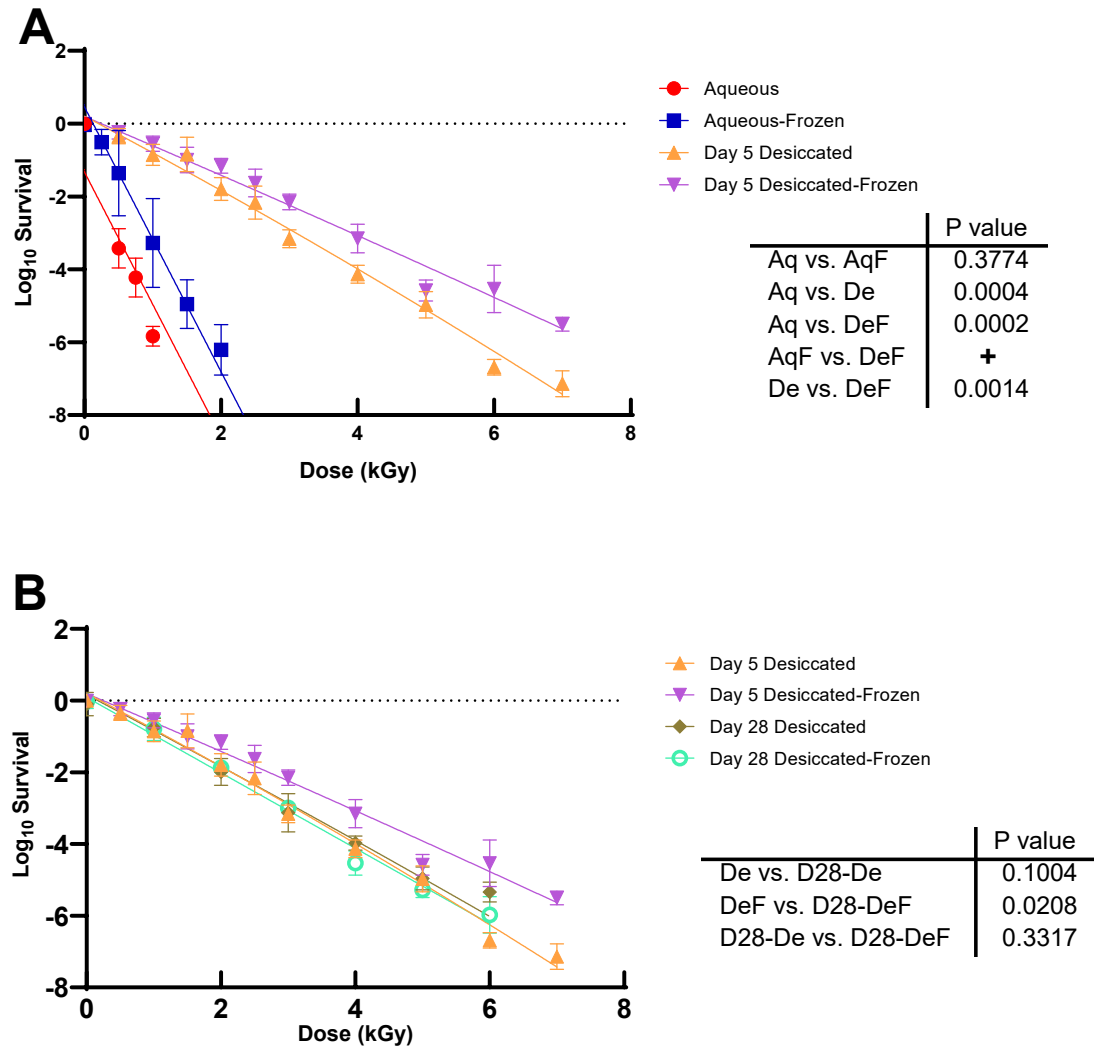
$$\frac{s(x)_{Day\ 5}}{s(x)_{Day\ 6}} = \frac{s(y)_{Day\ 5}}{s(y)_{Day\ 6}}$$

where $s(x)$ represents survival on a specific day at radiation dose x , and x and y represent different radiation doses, the following equation converting Day 6 values to Day 5 values was derived:

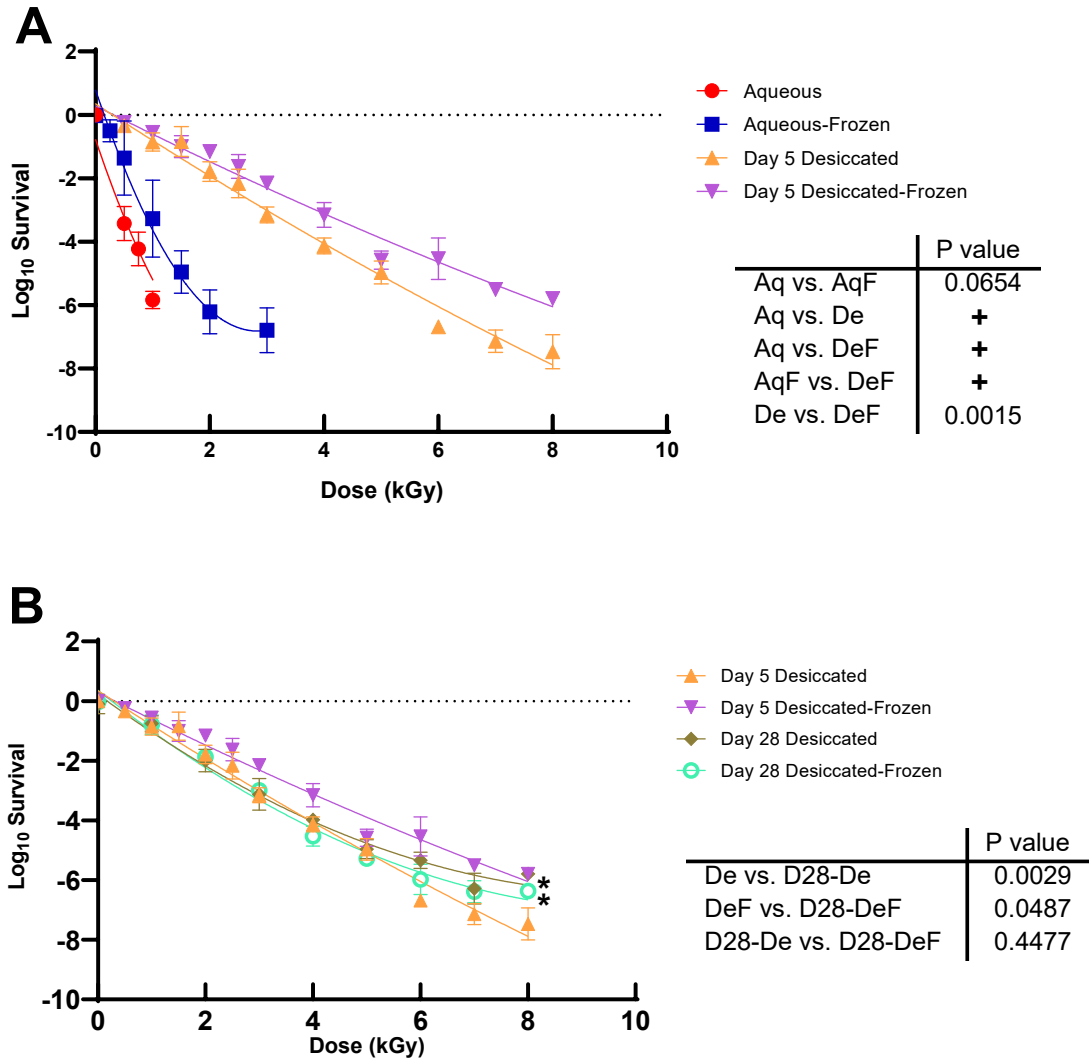
$$\log_{10}(s(x)_{Day\ 5}) = \log_{10}(s(x)_{Day\ 6}) + (\log_{10}(s(3\ kGy)_{Day\ 5}) - \log_{10}(s(3\ kGy)_{Day\ 6}))$$

where the Day 5 log survival value for a dose equals the Day 6 log survival at that dose plus the difference of the log survival values of Day 5 and Day 6 3-kGy doses.

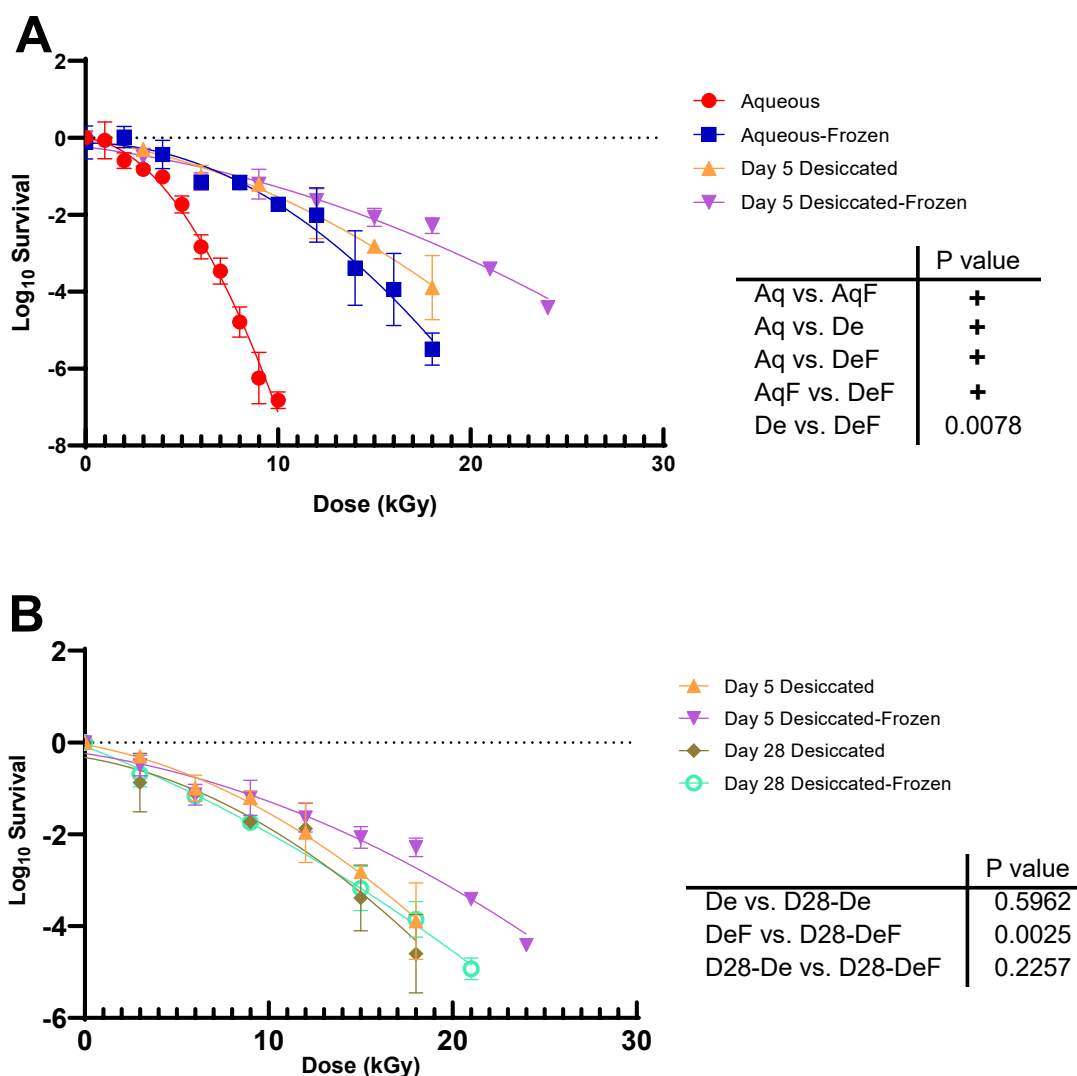
Supplemental Figure 10. *A. baumannii* Day 6 to Day 5 survival conversion equation for desiccated and desiccated-frozen samples.



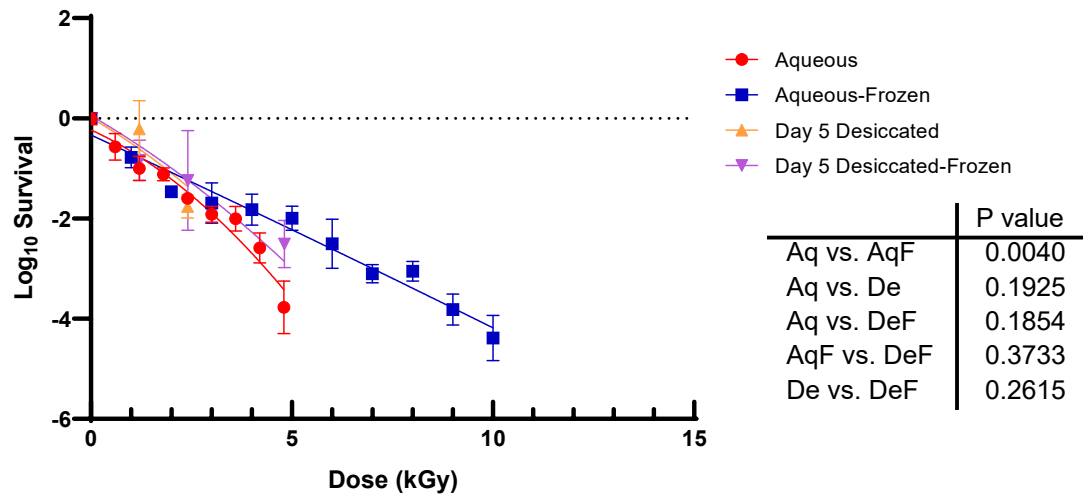
Supplemental Figure 11. *A. baumannii* survival to gamma radiation under different physicochemical conditions; B2 regression coefficients constrained to negative values. A. *A. baumannii* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *A. baumannii* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Significance threshold: $P < 0.05$. Results and SD error bars represent data from three replicates.



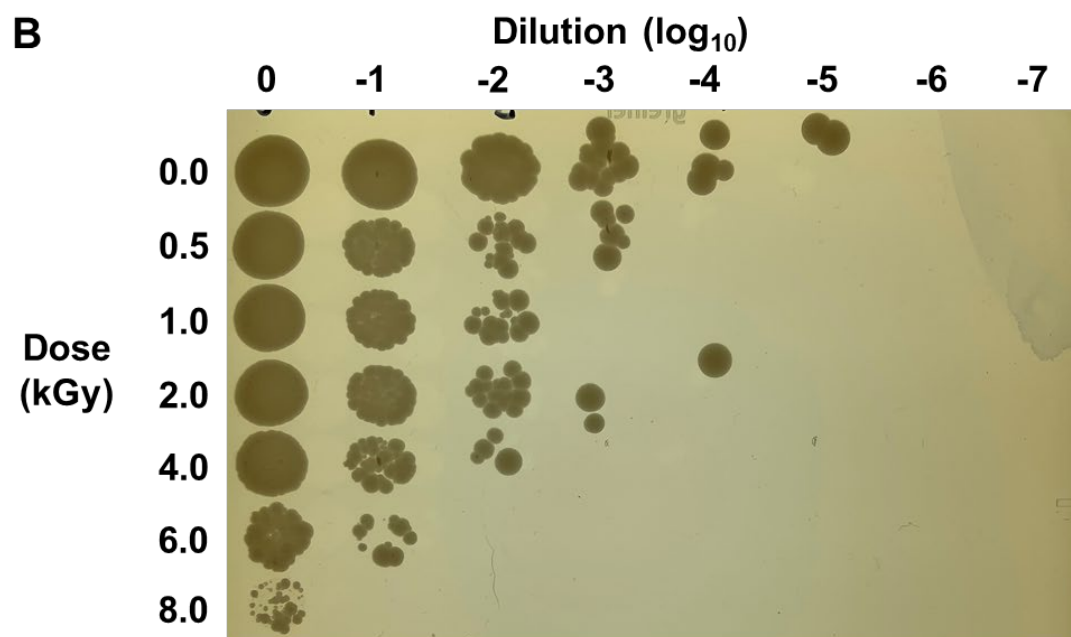
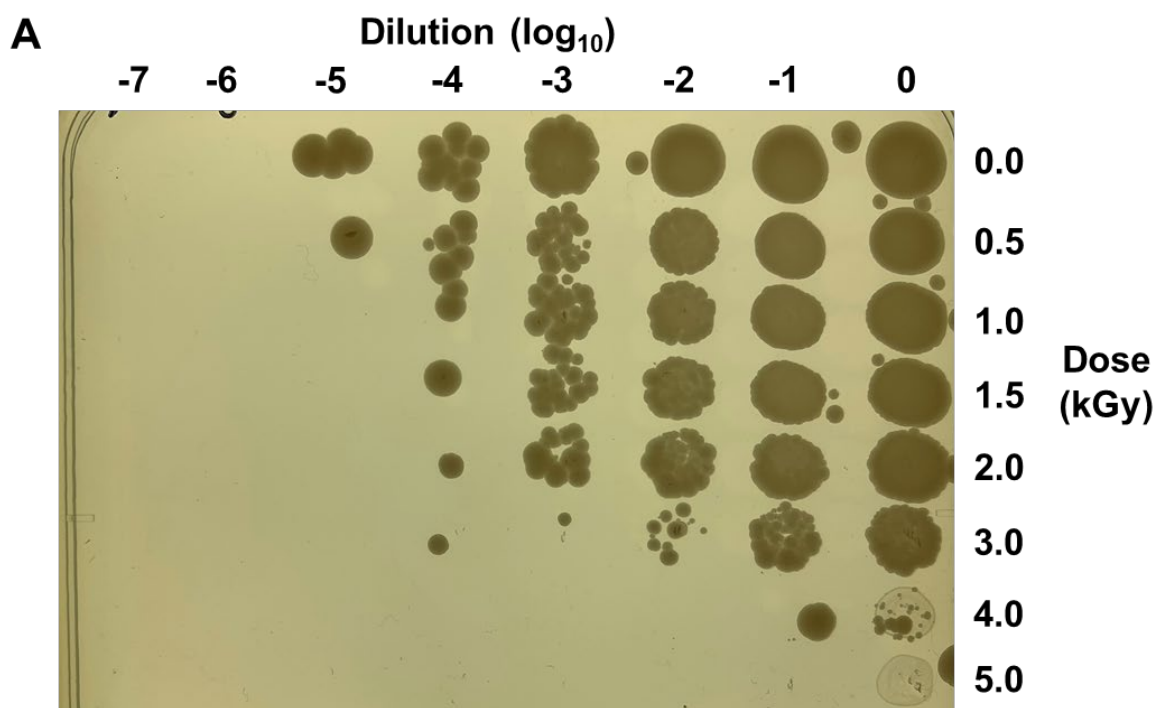
Supplemental Figure 12. *A. baumannii* survival to gamma radiation under different physicochemical conditions; all data points analyzed. **A.** *A. baumannii* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *A. baumannii* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$; *: single reading at this data point. Results and SD error bars represent data from three replicates.



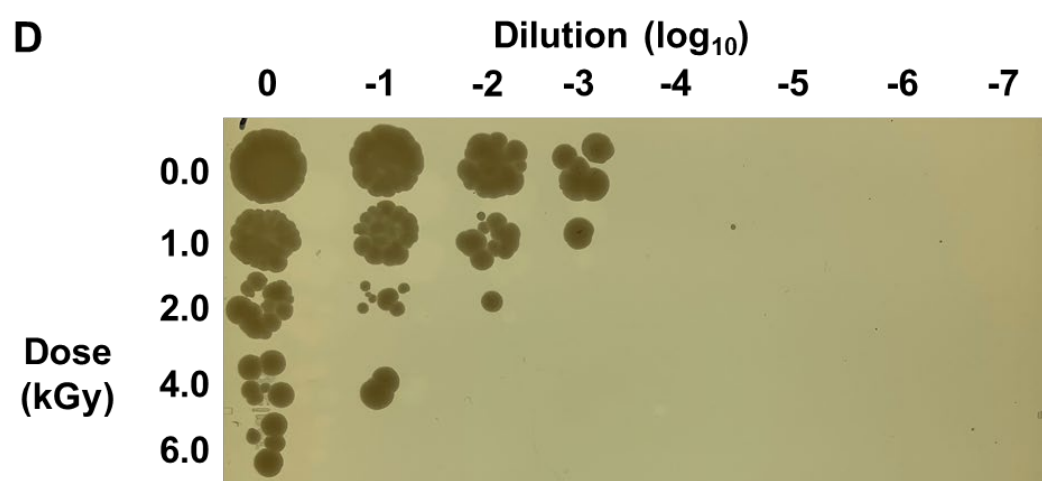
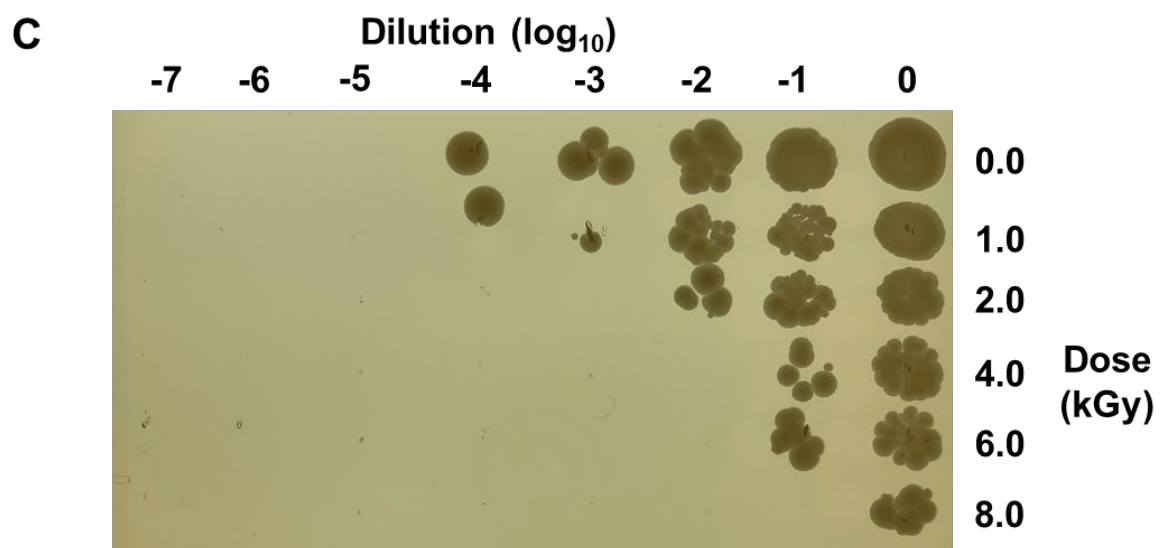
Supplemental Figure 13. *S. cerevisiae* EXF-6761 survival to gamma radiation under different physicochemical conditions; all data points analyzed. **A.** *S. cerevisiae* EXF-6761 survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *S. cerevisiae* EXF-6761 survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$. Results and SD error bars represent data from three replicates.



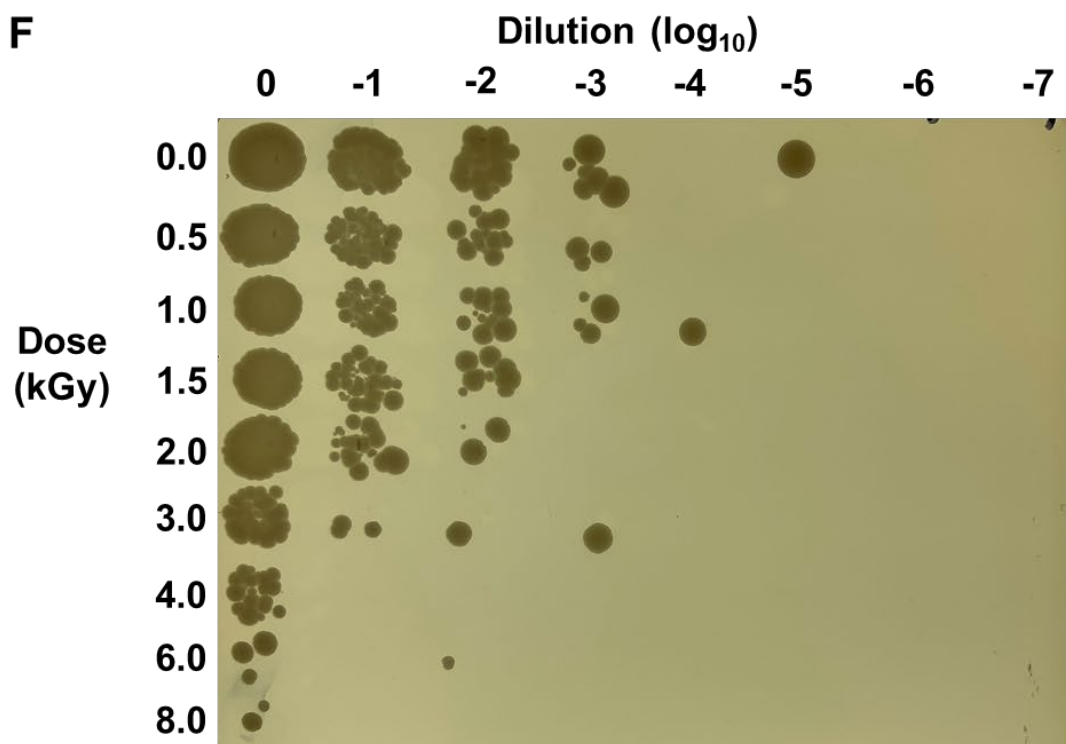
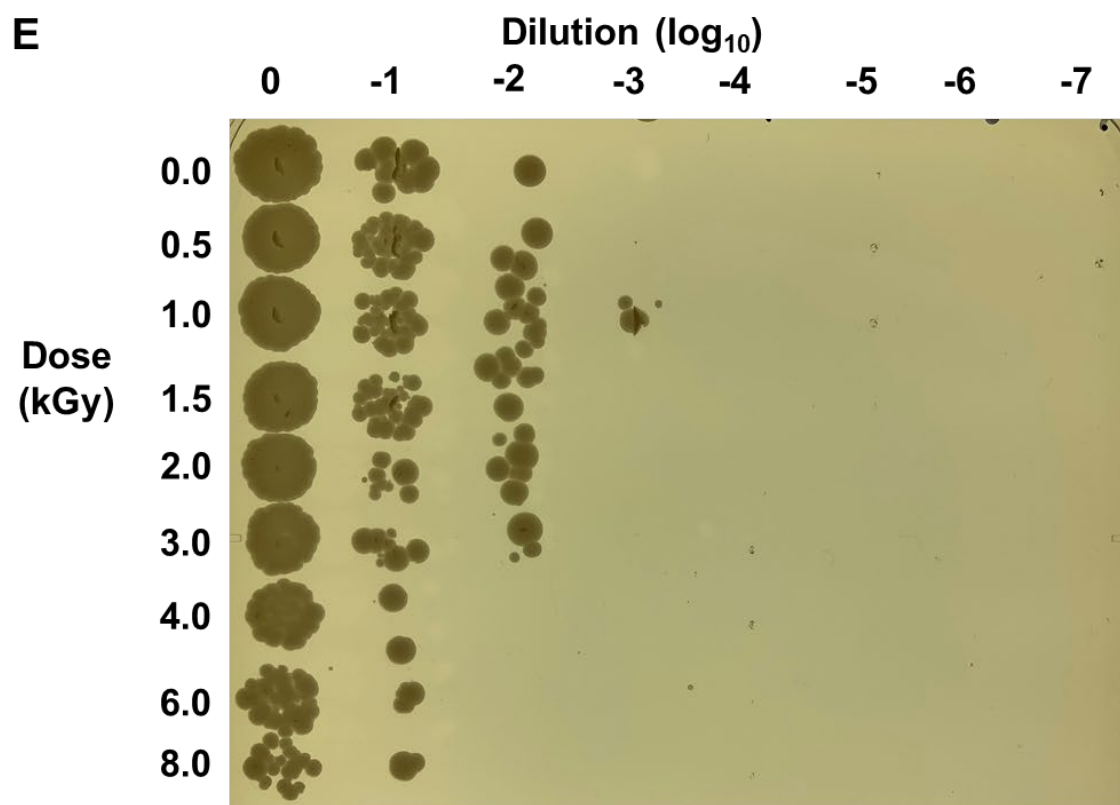
Supplemental Figure 14. *S. cerevisiae* EXF-6218 survival to gamma radiation under different physicochemical conditions; initial series. *S. cerevisiae* EXF-6218 survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C). Results and SD error bars represent data from two replicates.



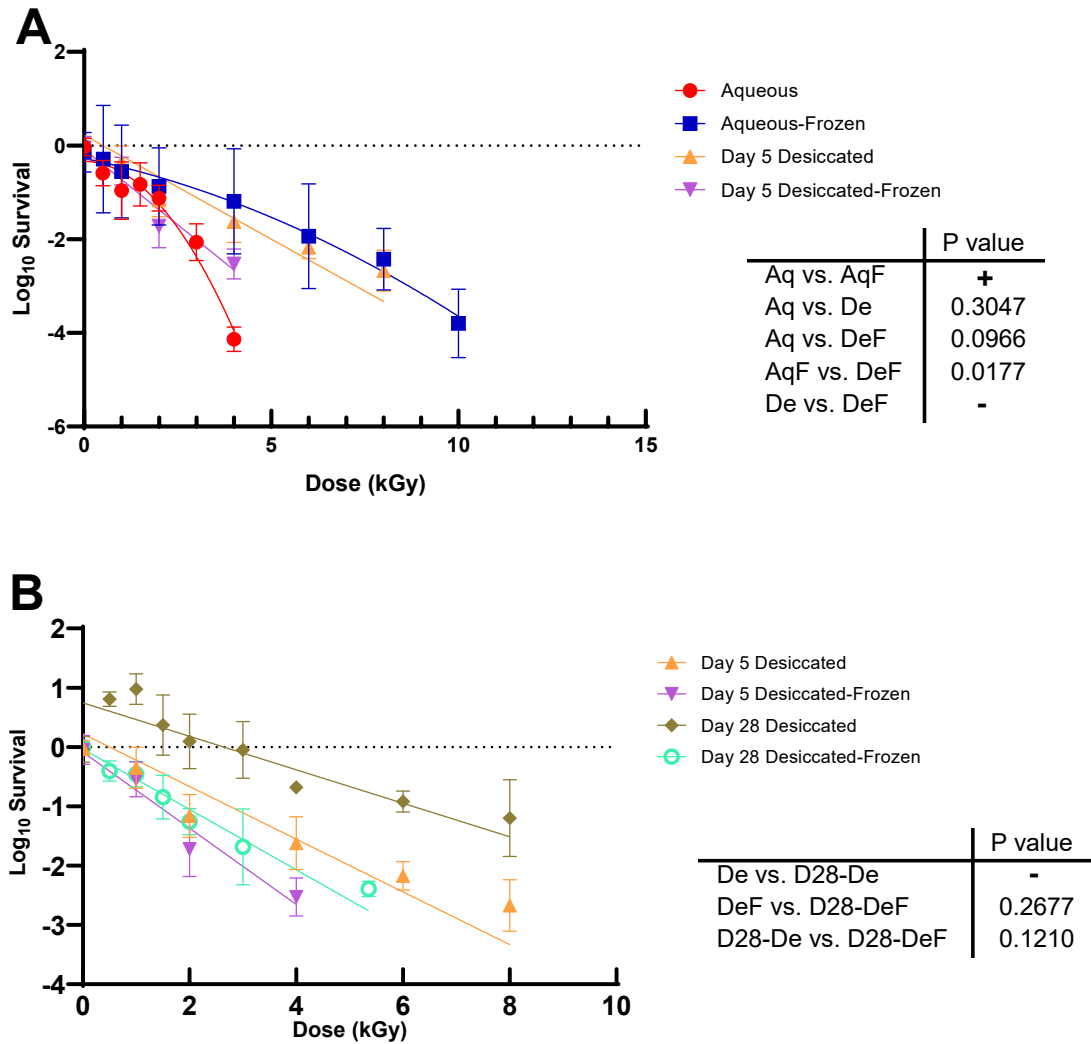
Supplemental Figure 15. *S. cerevisiae* EXF-6218 spot dilution survival assays. Photographs taken of each dilution assay plate for each cohort. Photographs are representative of two replicates. **A.** Aqueous. **B.** Aqueous-frozen. **C.** Day 5 desiccated. **D.** Day 5 desiccated-frozen. **E.** Day 28 desiccated. **F.** Day 28 desiccated-frozen.



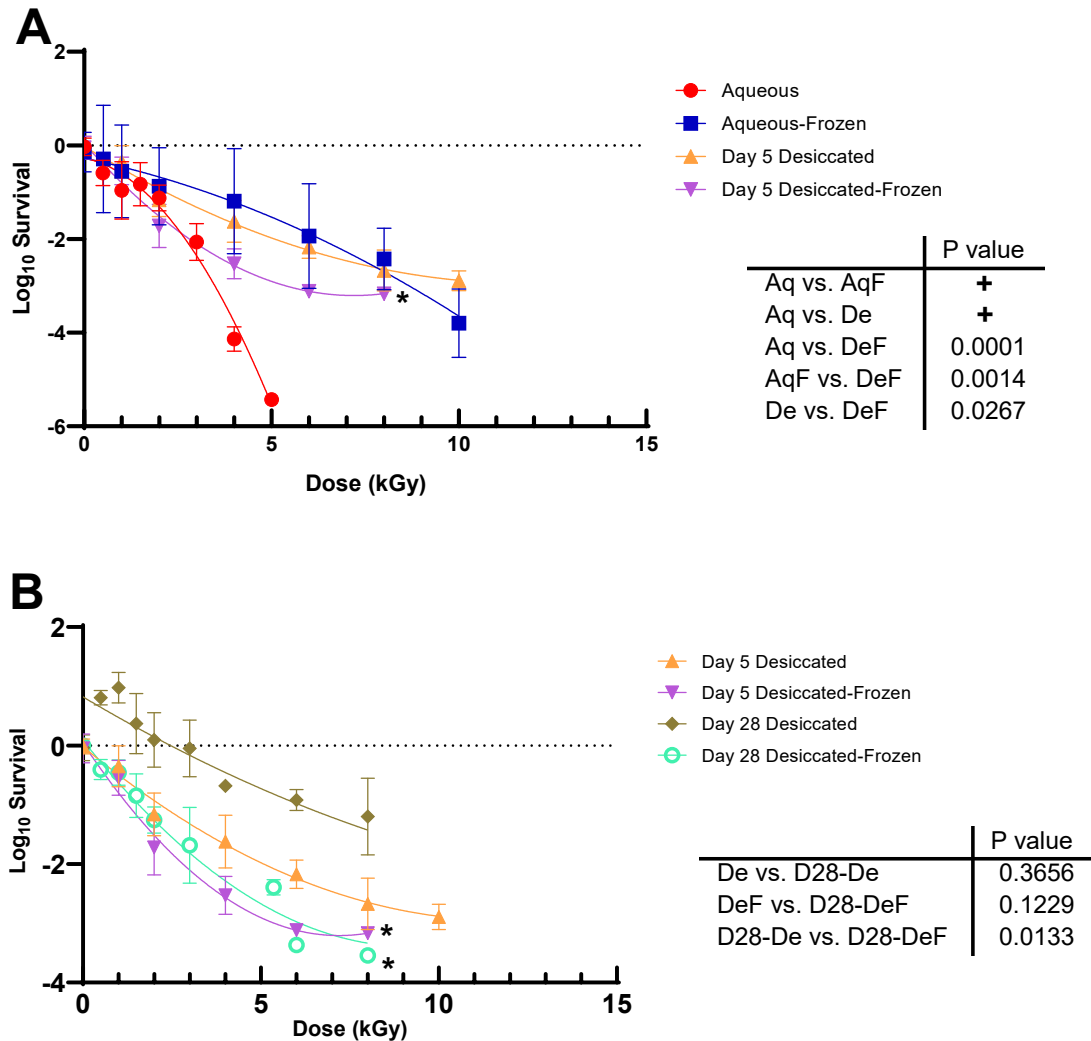
Supplemental Figure 15. *S. cerevisiae* EXF-6218 spot dilution survival assays (continued).



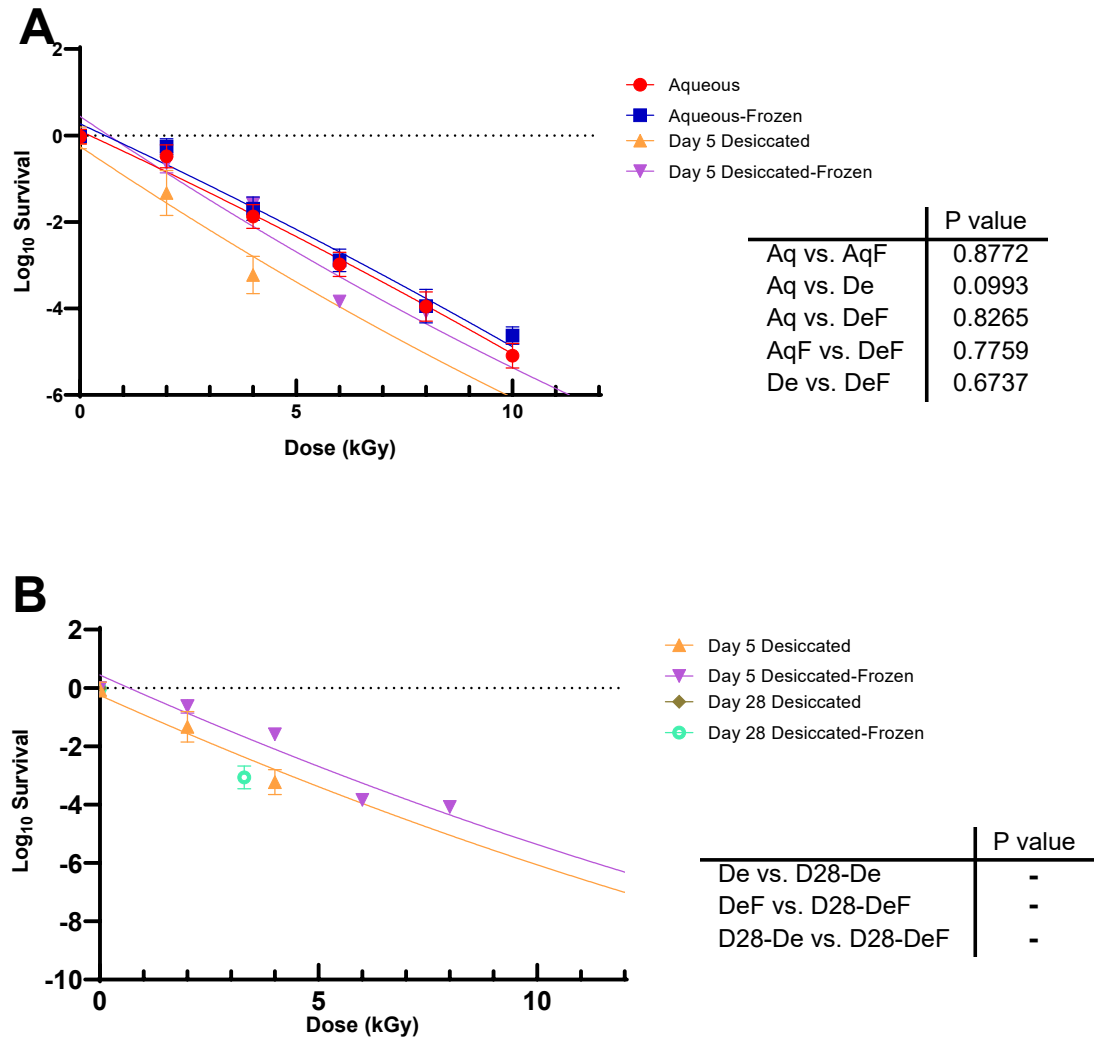
Supplemental Figure 15. *S. cerevisiae* EXF-6218 spot dilution survival assays (continued).



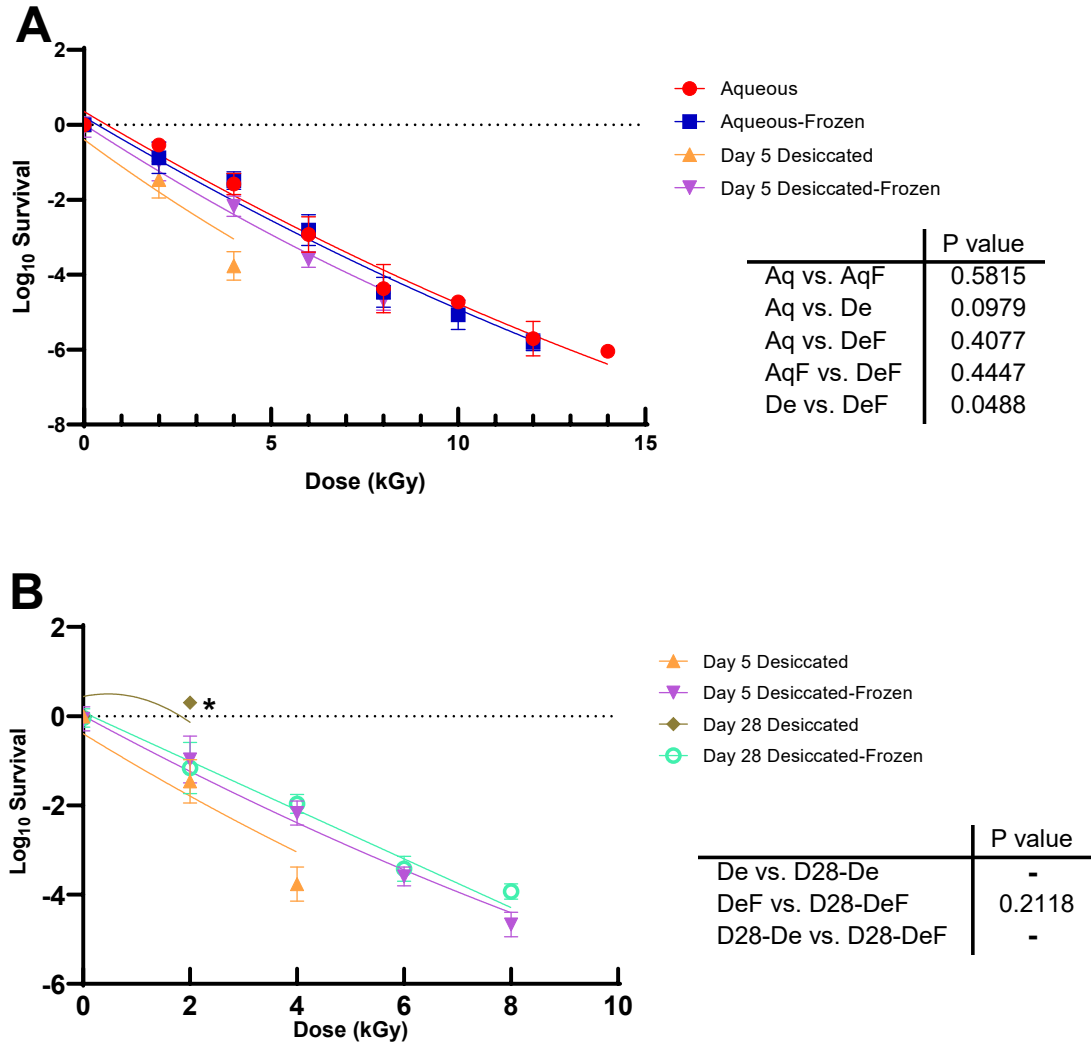
Supplemental Figure 16. *S. cerevisiae* EXF-6218 survival to gamma radiation under different physicochemical conditions; B2 regression coefficients constrained to negative values. **A.** *S. cerevisiae* EXF-6218 survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *S. cerevisiae* EXF-6218 survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$; -: unable to calculate. Significance threshold: $P < 0.05$. Results and SD error bars represent data from two replicates.



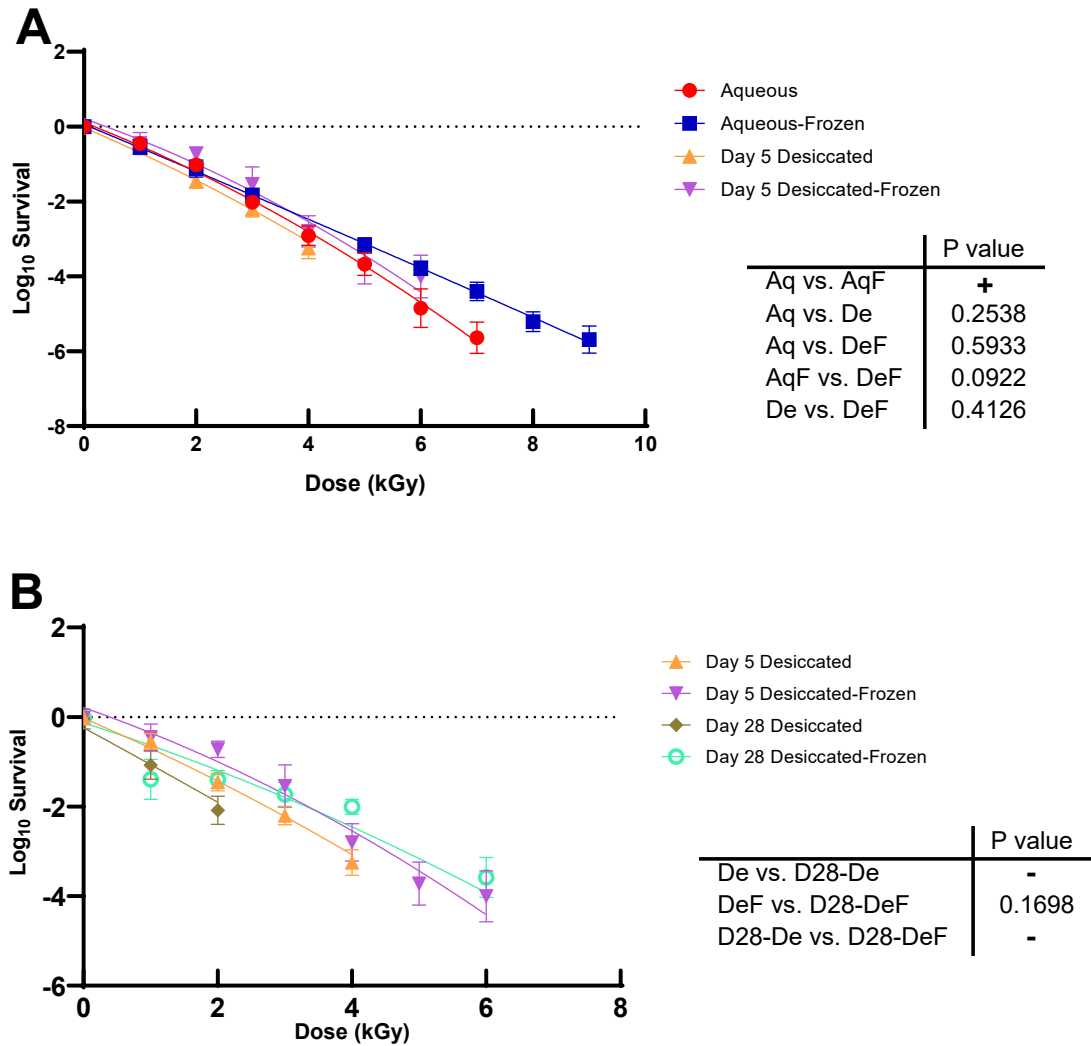
Supplemental Figure 17. *S. cerevisiae* EXF-6218 survival to gamma radiation under different physicochemical conditions; all data points analyzed. **A.** *S. cerevisiae* EXF-6218 survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *S. cerevisiae* EXF-6218 survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$; *: single reading at this data point. Results and SD error bars represent data from two replicates.



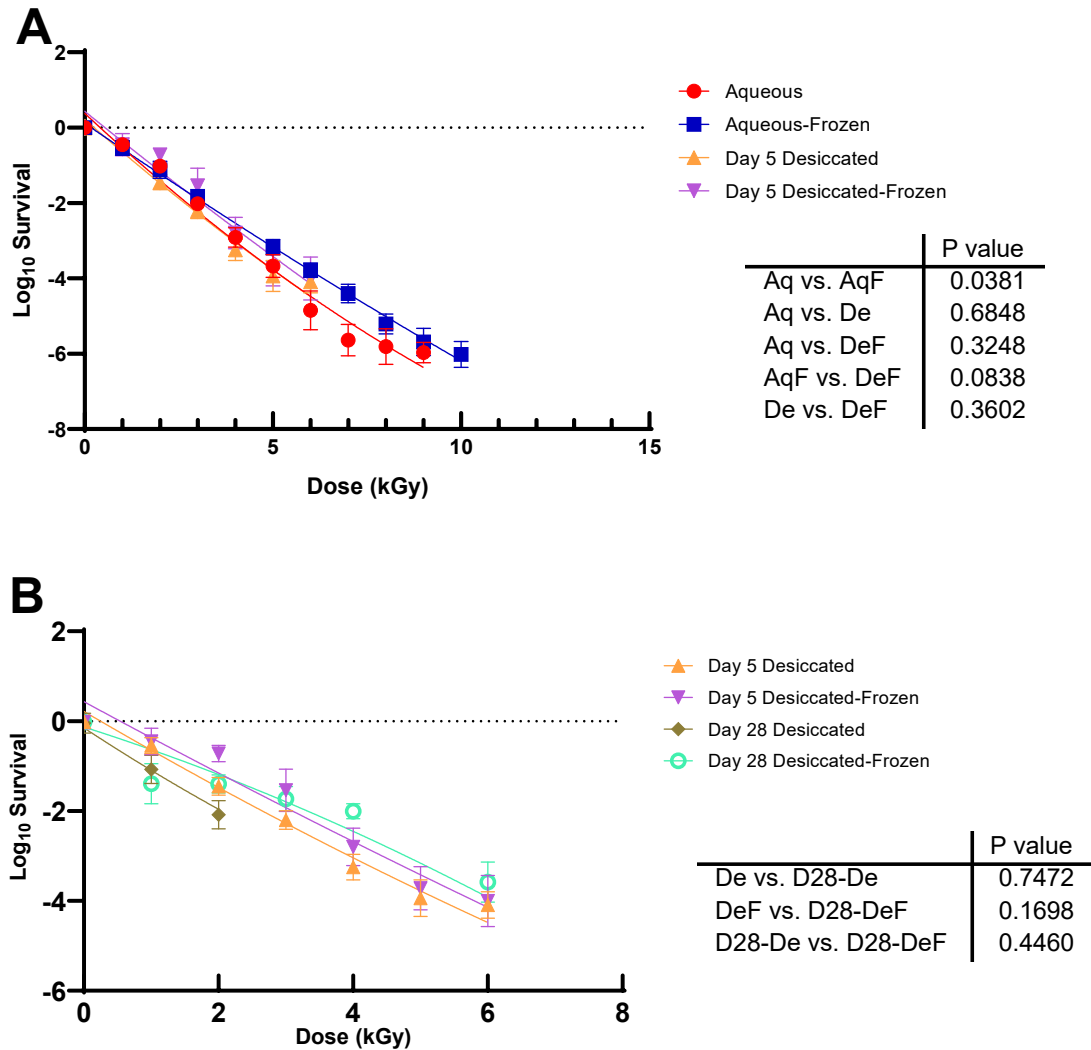
Supplemental Figure 18. *B. megaterium* survival to gamma radiation under different physicochemical conditions; all data points analyzed. **A.** *B. megaterium* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. megaterium* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). -: unable to calculate. Results and SD error bars represent data from three replicates.



Supplemental Figure 19. *B. thuringiensis* survival to gamma radiation under different physicochemical conditions; all data points analyzed. **A.** *B. thuringiensis* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. thuringiensis* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). *: single reading at this data point; -: unable to calculate. Results and SD error bars represent data from three replicates.



Supplemental Figure 20. *B. subtilis* survival to gamma radiation under different physicochemical conditions; B2 regression coefficients constrained to negative values. **A.** *B. subtilis* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. subtilis* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). +: $P < 0.0001$; -: unable to calculate. Results and SD error bars represent data from three replicates.



Supplemental Figure 21. *B. subtilis* survival to gamma radiation under different physicochemical conditions; all data points analyzed. **A.** *B. subtilis* survival curves for aqueous, aqueous-frozen, Day 5 desiccated, and Day 5 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. **B.** *B. subtilis* survival curves for Day 5 desiccated, Day 5 desiccated-frozen, Day 28 desiccated, and Day 28 desiccated-frozen conditions. Inset contains p-values for differences between survival curves. Aq: aqueous (0°C), AqF: aqueous-frozen (-79°C), De: Day 5 desiccated (0°C), DeF: Day 5 desiccated-frozen (-79°C), D28-De: Day 28 desiccated (0°C), D28-DeF: Day 28 desiccated-frozen (-79°C). Results and SD error bars represent data from three replicates.

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