JOHN D. CASTELLO
SCOTT O. ROGERS

Life in Ancient Ice

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LIFE IN ANCIENT ICE

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Edited by John D. Castello and Scott O. Rogers

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LIFE IN ANCIENT ICE

1

Introduction

John D. Castello and Scott O. Rogers

LIFE FLOATS in the clouds and is carried on the winds to all corners of the globe. This life includes fungi, protists, bacteria, archaea, viruses, pollen grains, and other biotic particulates that originate from the oceans, lakes, deserts, tropics, temperate regions, cities, forests, agricultural fields, and other habitats all over the world. Eventually these particles fall from the sky affixed to raindrops or snowflakes. Some die during their airborne journey, but many survive. If they fall to the earth in the polar regions they may become immured within the vast Arctic and Antarctic ice sheets for tens, hundreds, or thousands of years, and possibly longer. But is this the end of the story? No, it is not. Many of these life forms die, some become dormant, and still others thrive under such harsh but stable conditions. Eventually, the ice in which they are entombed melts, and these organisms, including the dead, dormant, and living ones, are released into the contemporary biosphere. This book is a compilation of the research that describes the environs and organisms that have been found in ice and permafrost.

What happens after the organisms reenter the contemporary environment? We do not know the answer to this question, but research to address this question must be conducted because some of the effects could be profound. Do these organisms reproduce and spread throughout the biosphere once again, or do they die because they cannot adjust to a different environment? If they survive, do they reintroduce ancient genes back into the contemporary gene pools? If so, what are the consequences of this gene flow on evolutionary rates, adaptability, fitness, and so on.? What are the consequences if some of these organisms are pathogens? Could they possibly start epidemics or a pandemic? These are just a few of the many questions that can be raised. The answers to them and others are unknown at this time, although the authors of the chapters in this book are addressing many of these questions.

This scenario may sound like science fiction, but it is not. Within this book is the evidence to support the entire story. It is a fascinating story. It is a new story because research into this field of science is new. Serious investigation began only thirty years ago in Russia with the work of Sabit Abyzov. It also is a very old story because it is likely to have been repeated annually since the vast ice sheets formed millions of years ago. As you will see, some problems

were solved, but others are yet to be solved. There were successes and failures; some questions were answered, but many more were raised.

This book had its origins in a workshop sponsored by the National Science Foundation and organized by the editors that was held at the Westin Salishan Lodge, Gleneden Beach, Oregon, from 30 June to 3 July 2001. The intensity and excitement were evident, indicating that additional collaborations and enhanced research activity should occur. The purpose of the workshop was to bring together experts studying ice, permafrost, ancient life, biological preservation, evolution, and astrobiology to assess current and future research that would extend our knowledge of life in ancient frozen matrices. Thirty-five internationally renowned scientists from Australia, Canada, Denmark, Germany, Israel, Russia, and the United States attended the workshop, and their presentations are the basis for the book. Many additional coauthors contributed to the chapters. The interactions among the participants were stimulating, and we hope that the workshop will lead to future meetings, collaborations, and research.

There were several objectives of the workshop, among which were

- providing the evidence for the presence of life in glacial ice and permafrost
- evaluating the reliability of that evidence
- discussing the significance and implications of the results to date
- identifying the more important and immediate research needs, and our recommendations for future research

The workshop and the book were organized according to scientific discipline. Chapter 2 introduces the many protocols that have been used when studying organisms and biological molecules entrapped in ice. It discusses some of the central problems, issues, and challenges in this research. One of the major challenges is to exclude, or somehow separate, external contaminants from the analyses of the internal ancient organisms and biological molecules. It recommends a set of goals to assure that only authentic entrapped organisms are identified as such. Chapters 3 through 6 outline the potential sources of microbes in polar environments, as well as describing microbes that each research group has found in ice, water, and the atmosphere. They state the quantities and types of microbes found in polar oceans, lakes, and the atmosphere, and describe some of the ways in which microbes can be transported to and deposited in glacial ice. In particular, waterborne and windborne (aeolian) transport is discussed in detail.

In chapter 7, Rivkina et al describe their evidence for microbial metabolic activity at temperatures below freezing. The authors state that the microbes do this by surrounding themselves with supercooled liquid water. This introduces the possibility that the microbes are not in states of suspended animation but, rather, may be metabolically active, although at low levels. This is an important

point, because if the organisms are able to survive for millennia or longer in the ice, they would probably need to continue to repair their DNA, since some damage to their chromosomes would be expected over such long spans of time.

The next several chapters (chapters 8 through 16) detail the microbes that have been found in permafrost (chapters 8 through 10) and glacial ice (chapters 11 through 16). Several describe fungi (chapters 8, 9, 11, and 12), many of which are common in cold climates; however, many others appear to have blown in from temperate and tropical zones. Similarly, many of the bacteria described (chapters 10, 15, and 16) appear to have been transported by wind from warmer climates. This is the case whether the glacier is near temperate zones (chapter 15) or very far from any temperate zone (chapter 16). Chapter 10 describes cyanobacteria that are able to survive and metabolize in cold and dark permafrost, which emphasizes that most of the viable microbes in permafrost and ice are able to survive in a range of environments, some of which are extreme.

Viruses are discussed in two of the chapters. In chapter 13, bacteriophage are described that were isolated from bacteria that had been isolated from polar ice cores. In a previous paper, the same authors described plant viruses isolated from ancient glacial ice. Together, these findings indicate two possible modes of preservation of viruses in glacial ice, the first being as intact virus particles (or fragments of particles), and the second is through integration into bacterial chromosomes (becoming prophage). In chapter 14, the significance of the disappearance and reappearance of viruses through time is discussed. The importance of glacial ice as a reservoir for these viruses also is discussed. In the future, this topic will probably be the focus of many research studies. The diversity of organisms described in these chapters, the extreme environments where they are found, the modes of movement, and the implications of their reentry into a more recent environment cause us to reexamine our views of where and how microbes can survive.

The final chapters describe several areas of research that have been developed over the past few years. The first of these chapters (chapter 17) presents details about Lake Vostok, a subglacial lake about the same size as Lake Ontario. The lake is buried by more than 3500 m of glacial ice and has been covered with ice for millions of years. Although Lake Vostok is the largest such lake found to date, there are hundreds of other subglacial lakes. No one knows whether life exists in Lake Vostok. It has been the subject of a great deal of speculation and will be the focus of research for many years to come. In the next chapter (chapter 18) a unique robot is described. This robot can be lowered into ice core boreholes to detect the presence of microbes. It can log data and send it to computers on the surface. It is possible that robots can be used more extensively to study other boreholes, as well as to examine subglacial lakes such as Lake Vostok. The following chapter (chapter 19) discusses the use of icy environments on Earth as model systems for the study of similar

environments on planets and moons. Ice has been found on our moon, Mars, and Europa (one of Jupiter's moons). Ice, permafrost, and ice-covered bodies of water on Earth are being used to test methods for detecting life on other bodies in the solar system. This chapter indicates one of the very practical aspects of the study of life in ancient ice. The final chapter (chapter 20) summarizes the most significant and important points in the book. It details some of the most key discoveries and outlines the areas that will be of importance in the future.

This book presents many of the facets of the study of organisms entrapped in ice. One can consider this a summary of the current state of research in the study of life, both contemporary and ancient, in ice. We anticipate that this will change rapidly in the near future. From the pioneering work of Sabit Abyzov and co-workers to the most recent publications, it is clear that ice and permafrost represent unique ecosystems and preservation matrices that can yield valuable information on microbial longevity, biological molecule preservation, past climates, recent climate change, evolutionary processes, epidemics, origins of life, life on other planets/moons, and other fields of study. It is true that with the scores of ice and permafrost cores that have been collected over the past several decades, we have gained a deep understanding of the potential benefits for the study of the ancient cryosphere. However, it also is clear that we have only begun to scratch the surface. At the time of printing, this is the only book of its kind, but we are hopeful that many more books on this subject will follow.

2

Recommendations for Elimination of Contaminants and Authentication of Isolates in Ancient Ice Cores

Scott O. Rogers, Li-Jun Ma, Yinghao Zhao, Vincent Theraisnathan, Seung-Geuk Shin, Gang Zhang, Catherine M. Catranis, William T. Starmer, and John D. Castello

VIABLE FUNGI, BACTERIA, AND VIRUSES have been isolated from glacial ice up to 400,000 years old and permafrost up to 3 million years old, originating from many geographic locales (1, 17–20, 25, 33, 40, 42). While many bodies of water contain higher concentrations of viable microbes than do deposits of ice, microbes encased in ice are usually in states of suspended animation or their growth is limited. This makes ice a unique repository of microbes frozen at various points in the past. Permanent ice covers approximately 10% (about 15 million square km) of the land area on Earth. Over 84% of this ice covers Antarctica. Another 14% covers land areas in the Arctic. The remaining 2% is in the mountains of Asia, North America, South America, Europe, New Zealand, and Africa (in descending order of percentage). Worldwide, the number of microbes entrapped in this ice is enormous. Recent microbiological studies of ice cores indicate that there are from 10^3 to 10^7 viable microbes entrapped l^{-1} of glacial meltwater (8, 18, 20, 31, 39, 40, 53, 56). At current rates of melting, this translates into an annual release of approximately 10^{17} to 10^{21} viable organisms worldwide. Spread evenly over the Earth, this represents approximately 10^8 to 10^{12} propagules $\cdot$ m^{-2}, or about 10^2 to 10^6 propagules $\cdot$ m^{-2} per species. Although these are estimates extrapolated from a handful of studies, they are indicative of the enormity of the releases of microbes from glaciers. Any global warming events would increase these numbers further. We have identified plant pathogens in the ice cores (17), but there are likely to be many other types of pathogens, including those that infect humans. While the number of organisms in ice worldwide is high and they are likely to be important ecologically, evolutionarily, and epidemiologically, extracting them from the ice for study is problematic.

Preservation of Ancient Specimens and Nucleic Acids

The primary reason that ancient microbes and nucleic acids are difficult to study is that they are always surrounded by recent contaminating microbes. Also, the ancient organisms are in relatively low concentrations, and they may have become damaged during their encasement in the ice. All of these factors make experimental manipulation difficult and replication impractical, in most cases. Nonetheless, there is an inherent interest in knowing how long microorganisms can remain viable, how long nucleic acids can survive, and what conditions are ideal for preservation. Theoretical expectations once had placed the limit for recovering viable bacterial spores at about 200,000 years (28, 57). Empirically based mutation rates that result in detrimental effects on spore viability have been used to estimate the half-life of *Bacillus subtilis* at 7000 years. Assuming an exponential death rate, a large population of viable spores would be detectable after several hundred thousand and possibly for several million years (28). These expectations assume that the immediate environment protects the spores from UV irradiation, oxidation, and other chemical damage. Studies on deep mud cores provide evidence for survival of thermoactinomycete endospores in excess of 1000 years (23). Viable microbes have been isolated from 320-year-old herbarium specimens (57). Thermophilic bacteria have been isolated from ocean basin sediment cores estimated to be 5800 years old (12). Bacteria also have been recovered from Roman archaeological sites that are 1900 years old (52), from Siberia up to 8000 years old (54), and from Vostok Station (Antarctica) ice cores, some of which are more than 400,000 years old (2–7, 17, 18, 33, 34, 37–40, 42, 48). Reports of the isolation, growth, and DNA characterization of bacteria from amber and halide crystals have pushed the theoretical limit to several hundreds of millions of years (15, 24, 58). However, this estimate has yet to be confirmed, because the isolations from the oldest specimens have not been independently replicated. Authenticity has been challenged in studies with amber, halide crystals, fossils, and others (10, 22, 36, Willerslev, Hansen, and Poinar, pers. comm.).

Currently, the most conservative estimates on the limits of DNA integrity range from 10^3 to 10^4 years for hydrated DNA within matrices at or near physiological temperatures (36, Willerslev, Hansen, and Poinar, pers. comm.). DNA is subject to chemical and physical damage (Table 2.1) by a variety of agents (35). In general, DNA is most vulnerable to degradation when hydrated, at low pH, and at elevated temperatures. Under these conditions, the purine and pyrimidine bases frequently are cleaved off the DNA, creating AP (apurinic and apyrimidinic) sites. Deamination of guanine, adenine, and cytosine occurs often at low pH (although deamination can also occur at high or neutral pH) and/or high temperatures. The sugar phosphate backbone can be broken by a

TABLE 2.1.
The most frequent damaging reactions to DNA

Original	*Reaction*	*Result*	*Pairs with*	in vivo *Repair*	*PCR / Sequencing Errors*
C	Deamination	U	A	+	+
^{5me}C	Deamination	T	A	–	+
A	Deamination	Hypoxanthine	C	+	+
G	Deamination	Xanthine	C	+	–
A	Depurination	No base	N	+	+
G	Depurination	No base	N	+	+
G	Oxidation	8-OH-G	A	–	+
A	Cyclic oxidation	8,5′ cyclic A	N	–	+
Py/Py	Dimerization	Dimers	None	+	+
N	Hydroxymethylation	H-N-CH_2OH	N	N	+

Notes: The result of each chemical reaction is indicated, as well as the change in pairing during replication, PCR amplification, and sequencing reactions; and whether the mutation causes a change in the resulting sequences. ^{5me}C indicates 5-methyl-cytosine, a variant of cytosine, which when deaminated becomes thymine. Py/Py indicates regions with adjacent pyrimidine residues (cytosine and thymine). The most frequent pyrimidine dimers that are formed are T-T, followed by C-T, T-C, with C-C being the least frequent. N indicates any nucleotide, A, G, C, or T.

number of agents, including ionizing radiation and alkalai (27). Oxidative damage can occur in a number of sites on nucleic acids. Aside from these major types of damage, other damage is caused by UV irradiation, as well as enzymatic and nonenzymatic attack. Thus, DNA will undergo less degradation under the following conditions: limitation of free water, neutral pH, limitation of oxygen, limitation of Mg^{2+} (a cofactor for many nucleases), and low temperatures. These conditions are present in many ice deposits.

Dry and cold conditions may extend the longevity of DNA to over 10^6 years (Figure 2.1). Previously, we reported the isolation of DNA from plant seeds up to 100 years old and from mummified specimens from 500 to over 45,000 years old (46, 50), in addition to viable bacteria and fungi, as well as fungal, bacterial, and viral nucleic acids, from ice cores 500 to 420,000 years old (17, 18, 37–40, 48). The average size of DNA from some 45,000-year-old mummified plant seeds was up to 3 kilobases, with some fragments up to 10 kilobases. This indicates that the limit of DNA longevity is well over 4.5×10^4, and could approach 10^6 years for desiccated tissues buried in soil and rock. Because viable organisms have been isolated from 420,000-year-old ice, longevity of DNA is expected to be greater for tissues embedded in these cold matrices, especially when enclosed within living organisms. When tempera-

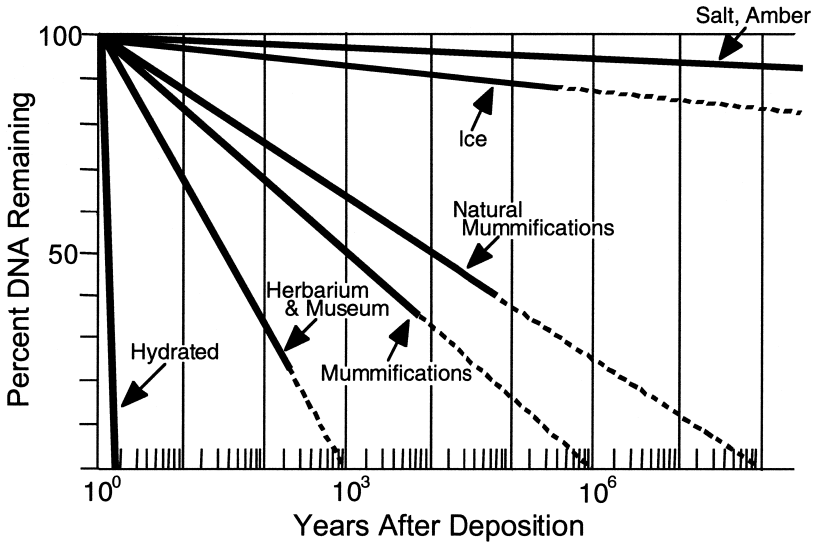

FIGURE 2.1. Representation of the amount of DNA remaining in ancient samples. The percentages are based on average molecular weights. Solid lines represent actual data, while dashed lines are extrapolations. Lines for ice, salt, and amber are based on isolations of living organisms (assuming that at least 90% of the genome is intact). Data are from the following sources: herbarium/museum—32, 45–47, and Rogers unpublished data; mummifications—41, 46, 51; ice—19, 20, 39, 40, 42, 59; amber/salt—15, 21, 24, 58. The results from salt, amber, and ice have yet to be independently confirmed by other laboratories.

tures are permanently below 0 °C, degradation slows down, because all of the degradative reactions (outlined above) are slower. Therefore, preservation of DNA in ice might exceed 10^6 to 10^7 years. Ice provides an environment that is cold as well as dry, in that free water is unavailable.

For any preserved specimens, the initial conditions are important (49). If the organisms or nucleic acids are deposited on the glacier already partially degraded, encasement in ice can slow further damage, but cannot reverse any prior damage. If the initial conditions are conducive to preservation of the organisms or nucleic acids, then there is a possibility that organisms and/or their nucleic acids can be recovered at a later time. It is clear that many types of organisms remain viable after being frozen for relatively short lengths of time. However, as indicated above, the upper limits of time for survival of the organisms and the macromolecules are currently imprecisely known. With larger organisms, the question is clearer than for microbes, because if a substantial number of vital cells die, the entire organism perishes. With microbes and nucleic acids the situation differs, because a part of the longevity is de-

pendent on population size. If the population size is large, then there is a greater probability that some of the members of the population will survive for extended periods of time. Limiting DNA degradation is vital to the survival of the organisms, but it is also important to researchers when attempting to obtain accurate sequences from DNA isolated from ancient matrices, such as glacial ice.

Methods to Study Microbes in Ice

Many protocols have been used to study and characterize microbes from ice cores (Figure 2.2). Light and electron microscopy have been used extensively to examine the meltwater for plants and animals (or parts thereof), as well as for microorganisms. However, classification to species is seldom possible with these methods. Culture methods also are widely employed, and have yielded a large number of isolates, many of which closely resemble described taxa. However, a large number may be new species. While culture methods are very useful, many microbes will not grow in culture. Estimates range from 0.1 to 17% of taxa that can be cultured. Therefore, culturing results provide a gross underestimate of the diversity of viable microorganisms in the ice.

Molecular characterizations are being utilized more frequently in studies of microbes from ancient ice. For cellular organisms, ribosomal DNA sequences are used to determine both taxonomic and genealogical affiliations. For viruses, a number of gene regions have been used. The DNA (or RNA for RNA viruses) is amplified using polymerase chain reaction (PCR). Then, sequencing is performed using the amplified product as the template. This process is used on the cultured organisms as well as on the meltwater, separately. The resulting sequences can be compared to sequences from contemporary specimens using various methods, including phylogenetic analyses. A major consideration with all of the methods, especially those that are very sensitive, is the avoidance of contamination.

Contamination and Decontamination

The elimination of all contaminating microbes and biomolecules is of primary importance in specimen authentication. Effective decontamination depends on the sterilization methods, sensitivity of assays, controls, replication of assays by independent researchers, and use of indicator organisms and nucleic acids. Contamination is always present on the outside of the core sections. The contaminants originate from the drill, drilling fluids, handling, transport, storage, and final processing of the ice core sections. Attaining sterility at the field coring sites, and during subsequent transport to the storage and research facili-

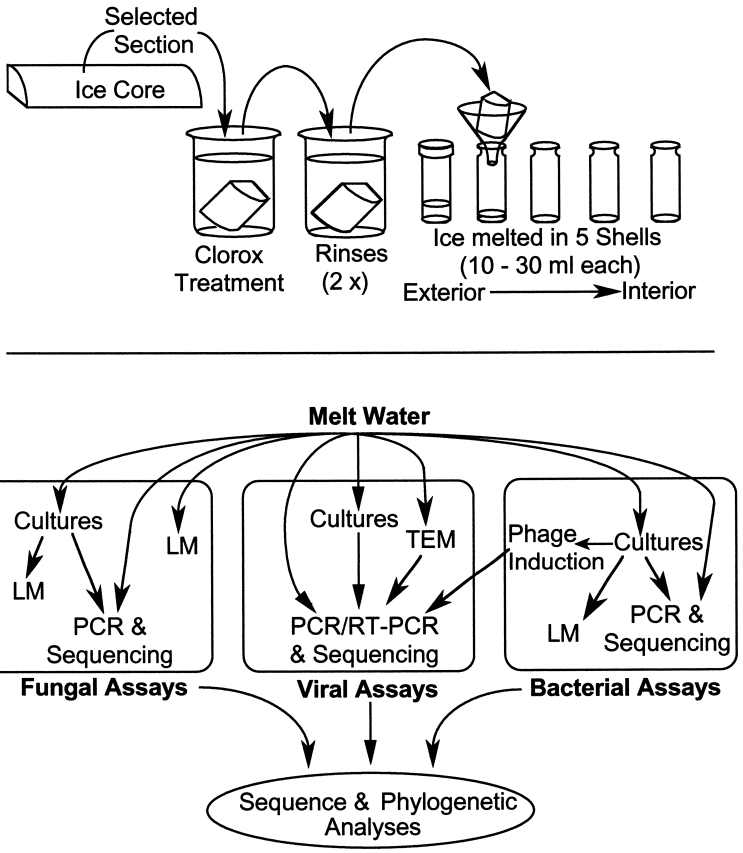

FIGURE 2.2. Flow diagram of decontamination, melting, and assay protocols. Upper portion shows the decontamination and melting protocol. Lower portion indicates the various assays for viable microbes and nucleic acids. TEM = transmission electron microscopy, LM = light microscopy, PCR = polymerase chain reaction, RT-PCR = reverse transcription-PCR.

ties, is impossible. Once the core is transported to the laboratory for study, sterility is possible, but the outer surfaces of the core section must be carefully and effectively decontaminated and/or removed. During culturing and molecular biology procedures, sources of contamination may include ventilation systems, work surfaces, culture media, reagents, researchers (hands, hair, breath, etc.), and others. In addition, contamination may have entered the core sections through minute cracks and breaks in the ice. Once in the laboratory, all sources of contamination must be eliminated, and the core must be decontaminated prior to assays for microbes and/or biomolecules.

Comparisons of Decontamination Protocols

There are two major objectives of any decontamination protocol. The first is to ensure that contaminants are completely eliminated. The second is to protect rare and/or sensitive organisms and nucleic acids inside the ice cores during decontamination and melting of the ice. Some of our research efforts have dealt with solving these important problems. Synthetic, or "sham," ice core sections (a cylinder 5 cm in diameter and 15 cm in length) have been produced in our laboratory, consisting of frozen suspensions of specific indicators on the interior (*Aspergillus tereus* spores and DNA, *Aureobasidium pullulans* spores, *E. coli* cells, and tomato mosaic tobamovirus [ToMV] virions), and other indicators spread onto the exterior (*Ulocladium atrum* spores and DNA; *Bacillus subtilis* spores; and lambda phage DNA). We evaluated UV irradiation, liquid sterilants (solutions of sodium hypochlorite, ethanol, hydrogen peroxide, sodium hydroxide, and hydrochloric acid), as well as ablation (removal of outer layers with sterile distilled water or sterilized razor blades) and mechanical methods (sterilized drills, saws, and heated probes) to extract inner core regions (Tables 2.2 and 2.3). Then, the treated cores were melted in shells, where shell 1 was the outermost part of the core and shell 5 was the innermost portion (Figure 2.2, upper). Each shell was approximately 5 to 10 mm in thickness, yielding approximately 20 ml of meltwater each. The treatment that assured complete elimination of all outer organisms and nucleic acids and preserved the largest proportion of the inner core regions was a 10 second exposure to a solution of 5% sodium hypochlorite (Table 2.3).

While UV irradiation was initially promising, we discovered that some microbes (most notably, *Ulocladium atrum*) are extremely resistant to treatment by this method. For example, 100% of the cultures of *Penicillium commune* were killed by a dose of 27, 540 $J \cdot m^{-2}$ of 265 nm UV irradiation (germicidal lamp placed 2 inches from the culture for 5 minutes). However, cultures of *U. atrum* withstood twelve times this dose with no notable effects (100% of the cultures survived with no effects on numbers or sizes of colonies). Three protocols (95% ethanol, heated probe, and drill extraction) all produced similar results. External contaminants were found through shell 2, and there was a reduction in the number of inner microbes and nucleic acids in shells 1 through 3. All three methods utilized a treatment of 95% ethanol, which might partially explain the similarity of results. While ethanol will kill many microbes, it is ineffective toward others and does not destroy nucleic acids. Thus, it was relatively ineffective. Treatment with hydrogen peroxide (6% or 35%) and sodium hydroxide/hydrochloric chloride (1N or 10N each) produced similar results. In each case, external contaminants were evident in shells 2 and 3, respectively. Additionally, the quantities of internal microbes and nucleic acids were reduced all the way into the innermost shell. With