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JOURNAL OF THE AMERICAN BIOLOGICAL SAFETY ASSOCIATION

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VISION

ABSA, the leader in the profession of biological safety.

MISSION STATEMENT

The American Biological Safety Association is dedicated to expanding biological safety awareness to prevent adverse occupational and environmental impact from biohazards.

GOALS

- Expand professional and public awareness of biological safety through effective communication.
- Participate in the development of biological safety standards, guidelines, and regulations.
- Develop ABSA as the recognized resource for profession and scientific expertise in biological safety.
- Advance biological safety as a scientific discipline through education, research, and professional development.
- Develop and maintain standards for biological safety professionals.
PRESIDENT'S PAGE

Have you ever thought about the impact ABSA has had on your position as a biological safety professional, on the way you are perceived in your facility as a biological safety professional, or on how you are seen in relation to your peers in the environmental health and safety field? The ABSA Council and committee members have invested their time and efforts over the years toward building upon the organization's professional image, and the most recent efforts have been the institution of the credentials for the Registered Biosafety Professional (RBP) and the Certified Biological Safety Professional (CBSP). These credentials give ABSA, and the professional, the recognition needed within the scientific community. We have always wanted our peers to associate ABSA, and our profession, with the highest standards in biological safety, and providing credentials has enabled us to meet this goal.

Does the value of the RBP and the CBSP influence an employer in hiring an employee or in choosing a consultant? I have polled several Environmental Health and Safety directors, and every one of them have said that it was important to them to hire a person with a certification or credential in the desired field. (Other factors being considered, such as experience.) If an industrial hygienist position was available, the employer would look favorably on the applicant with a CIH; in hazardous materials, a CHMM; in physical safety, a CSP; and in biological safety, a RBP or CBSP. The reason would be because these credentials indicate that a person had taken related educational courses, and had shown competency in a field of work. In the biotechnology industry, the preferred certifications would differ from the ones needed in a large university research facility. The CSP may be more valuable as a general safety certification in an industry where the safety staff must wear several hats, but in a university, the CBSP, CIH, and CHMM would be preferable since the staff is usually dedicated to one field of expertise.

Our job then remains to make the segment of the industry that is outside of our usual customers knowledgeable of the RBP and CBSP credentials and their inherent value. I encourage you to use the RBP and CBSP in order to get as many people as possible to talk about our biological safety credentials. Your co-workers and customers should be made aware of your specialized knowledge so they can, in turn, pass this information along to members in their organizations. We will then have planted the seed of information in people's minds, and therefore, customers needing our help will have a better chance of being made aware of our expertise and of the quality of services we can provide.

Marilyn Misenhimer
University of Southern California
Los Angeles, California
EDITOR’S PAGE

Ring out the old, ring in the new. An appropriate saying for the New Year and for the appointment of a new Editor for the *Journal of the American Biological Safety Association* (JABSA). Past President Richard Knudsen has graciously accepted the post of Editor starting with the first issue for 1999. This is a very important and portentous appointment for the continuing welfare of the Association. Not only has his knowledge and competence been recognized by the Association and certified by the membership through his election to President but having served in that office he is now intimately acquainted with all the workings and aspirations of the Association and has had multiple opportunities for interactions with the membership at large. Starting from this base, Dr. Knudsen has formulated many new and innovative plans for JABSA that I find very exciting in prospect. I know I speak for the entire Association membership as well as myself when I say, “We wish you well and lots of success in building the Journal to the eminence it has the potential to achieve.”

For myself, I can say it has given me great personal and professional satisfaction to have had the opportunity to participate in the founding and nurturing of a brand new scientific journal, especially as this one is unique—no other like it exists. In addition, in spite of occasional frustrations with overdue deadlines and shortages of publishable papers, it has been a joyful experience through my contacts with the members of the Editorial Committee, the Associate Editor, Linda Martin, and the many authors, some old, dear friends, some new, whose papers have appeared in the several issues of JABSA over the past three years. My special thanks goes to two who have done the hard work of getting the journal produced and into the hands of Association members: Karen D. Savage, JABSA Production Editor, located in the Association’s National Office and Constance Smith, my Administrative Assistant.

I am positively delighted to have Richard Knudsen take over the Editorship of the journal, someone young, vigorous, and knowledgeable about the Association. It was what I planned for from the start and hoped would occur. May success and achievement be his in this new post.

Melvin W. First
Harvard School of Public Health
Boston, Massachusetts
ABSA: THE PAST, PRESENT, AND FUTURE

Richard C. Knudsen
Centers for Disease Control and Prevention, Atlanta, Georgia

The Past

The origins of the American Biological Safety Association are associated with the biological warfare (BW) program that was developed at the U.S. Army Biological Research Laboratories at Camp (now Fort) Detrick during World War II (1943-45) (Bernstein, 1987, Covert, 1993, Kruse, 1998). Two months before Pearl Harbor, Secretary of War Henry L. Stimson asked the President of the National Academy of Sciences to evaluate the requirements for a biological warfare (BW) program. In February of 1942 a special committee of the National Academy of Sciences submitted a report to Mr. Stimson saying that an enemy attacking with biological weapons could gravely harm crops, livestock and human beings and made recommendations for the future of the BW program. As a consequence Camp Detrick was established in 1943 to serve as the focal point for developing America’s offensive and defensive BW program. Biological warfare agents are, by their very nature, highly hazardous and the successful development of this program needed to ensure the safety of the scientists and technicians working in this program, as well as the safety of the surrounding community of Frederick, MD. At this time there was no disciplined body of knowledge applicable to working safely with BW agents during their laboratory development, pilot plant production and testing. Out of necessity Camp Detrick personnel proceeded to develop the appropriate safety facilities, equipment and practices and in the process gave birth to the field of biological safety (Covert, 1993). Dr. Gail Dack was the first Safety Director. Dr. Arnold G. Wedum, widely recognized as the father of biological safety, became Safety Director in 1946.

By 1955 interest in further developing the concept of “biological safety” and sharing information on this subject with other practitioners led Dr. Wedum to convene the 1st Biological Safety Conference at Camp Detrick. Fourteen representatives from Camp Detrick, Pine Bluff Arsenal, Arkansas, and Dugway Proving Grounds, Utah attended. The 1st Biological Safety Conference evolved into the Annual Biological Safety Conference and has met every year since 1955. Since the first conference biological safety has evolved from being concerned with BW agents into applying to all biological agents in a wide variety of laboratory and non-laboratory settings. In 1998 we held our 42nd Annual Biological Safety Conference at Orlando, FL with more than 300 attendees from academia, industry and government throughout the United States and the world. Manny Barbeito and Richard Kruse, original members of Dr. Wedum’s safety group, have documented the early biosafety conferences in a series of JABSA articles (Barbeito and Kruse 1997a, 1997b, 1998). Other milestones in the development of the field of biological safety include the establishment of the position title “Biological Safety Officer” in the 1976 NIH Recombinant DNA Guidelines (Federal Register, 1976) and the evolution of the Annual Biological Safety Conference into the American Biological Safety Association (ABSA) in 1984. This year also marked the publication of the first edition of the CDC/NIH Guidelines “Biosafety in Microbiological and Biomedical Laboratories” (BMBL) (Richardson and Barkey, 1984) which provided for the first time a disciplined body of information on biological safety. The seeds that were planted by the personnel at Camp Detrick in 1943 had grown into the “biosafety tree” in 1984.

By 1991 ABSA had grown to the extent that part-time management of the organization by members with full-time career positions had become too time consuming and a professional management organization, Stygar Associates, was hired to ensure the proper operation of the business side of ABSA.

This article is an expanded version of the President’s Address from the 42nd Annual Biological Safety Conference held in Orlando, Florida on October 25-28, 1998.
A growing professional organization needed a newsletter to keep its members informed (the Internet was in its infancy in 1991) and biological safety as a profession also needed training programs for its members. Both of these were initiated by ABSA in 1991. Members felt that they needed to be recognized as professionals and in 1994 the Registered Biological Safety Professional program was established. By 1996 ABSA members felt that the profession of biological safety had matured enough to initiate the first issue of the Journal of the American Biological Safety Association (JABSA). National and international communications via the Internet were growing rapidly during the early '90s and ABSA's Internet site was established in 1996. Further growth of biological safety as a profession occurred in 1997 with the initiation of the Certified Biological Safety Professional program in association with the American Society of Microbiology's National Registry of Microbiology. This year also marked the first ABSA sponsored spring training program in association with the Eagleson Institute.

The Present

Our members can be proud that ABSA has now established an ambitious program of annual meetings, biosafety training, professional certification, and a variety of ways of communicating with fellow members and the public through our newsletter, journal, other publications, and our Internet site. In 1998 we co-sponsored with the Centers for Disease Control and Prevention the 5th National Symposium "Rational Basis for Biocontainment." We co-sponsored with the Eagleson Institute our second spring training session on animal biosafety and R-DNA. We also published our first book "Proceedings of the 5th National Symposium" and our training guide. We added a list of infectious agents classified by hazard to our web site. We will also update our web site in December 1998 and turn it over to professional management in 1999. Our annual meetings continue to attract top notch speakers from government, industry, and academia. This year Dr. Joanne Burkholder of North Carolina University, author of "And the Waters Turned to Blood" told us about _Pfeisteria piscicida_, Thomas Rowe of CDC reviewed the avian influenza outbreak of 1997-98, Nancy and Jerry Jaax of USAMRIID at Fort Detrick shared their experiences in the hot zone with us, and Margaret Race of NASA told us about "Extraterrestrial Sample Return."

In 1998, in accordance with our strategic plan, we established a Marketing Committee and a Media Committee to help us show our wares to the scientific community and to the public. We developed a Technical Resource Committee to focus attention on the resources that we can offer to the public. We developed a Publications Committee to oversee and develop ABSA publications of which several are in development. These new initiatives should be showing results over the next few years.

The Future

Our future is going to involve more people on this planet and tremendous increases in technology. For ABSA this means that our challenges of the future will lie in the areas of more emerging and re-emerging biological agents of plants, animals and humans, the possible use of biological agents for terrorism, safely applying biotechnology in the laboratory, ensuring the safe application of biotechnology to the environment, and taking advantage of all the new advances in electronic communications to advance ABSA's mission. I have discussed these future challenges in more detail in the following paragraphs.

Our world population is increasing by 70 million people a year. To support such huge increases in people more land needs to cleared and food and water supplies increased. Social, economic, and political systems need to absorb them. More people means increased use of fossil fuels which leads to global warming which in turn may alter or broaden the geographic range of insect vectors for diseases such as dengue and malaria. As forest land is cleared people are brought into contact with new agents such as Sabia virus and new vectors which have been hidden in the forest (Gibbons, 1993). Modern travel allows people, as well as well as hitch hiking infectious agents such as influenza viruses and insect vectors to be transported around the world in a day. As more people crowd into urban areas which cannot support them the poverty level increases and infectious agents, such as measles, have greater opportunities to spread. In a 1997 review of emerging diseases, Mahy (1997) listed 42 new viruses and 4 new rickettsia discovered since 1988. Other new infectious agents whose names
were unknown 20 years ago but with which we are now familiar include: human immunodeficiency virus, the causative agent of AIDS; Hantavirus, the causative agent of pulmonary syndrome; *Helicobacter pylori* as a causative agent of peptic ulcers; Chlamydia pneumoniae as a causative agent of atherosclerosis; *Escherichia coli* 0157:H7 as a foodborne pathogen causing hemolytic uremic syndrome; *Cryptosporidium parvum* as a causative agent of waterborne intestinal disease; *Pfiesteria pscacida* toxin as a causative agent of skin ulcers and neurological diseases; and obscure agents that cause transmissible bovine spongiform encephalopathy. Other emerging or reemerging diseases include antibiotic resistant *Mycobacterium tuberculosis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*. The global public health issues associated with emerging infectious diseases has spawned a new journal *Emerging Infectious Diseases*. As a result ABSA should expect a continuing increase in new infectious agents, new or wider ranges of vectors, and the reemergence of long subdued infectious agents.

Fifty-five years ago the development and use of an infectious or biological agent for biological warfare purposes was a technologically complex and costly process that could only be performed within well financed and staffed government laboratories. Today's advances in technology and information have made these agents relatively easy to grow, cheap to produce, and easy to disseminate. Information available on the Internet provides "how to" instructions. Biological agents have been called the poor man's weapon of mass destruction because they can be produced in the basements of our homes. Alternatively, deadly agents may be purchased from supply houses or stolen from containment laboratories. In recognition of this the federal government has restricted the transfer and use of the worst of these agents, termed "select agents," in a new federal regulation (Federal Register, 1996). The growth of the field of genetic engineering also raises the specter of developing new bioterror agents from genetically modified microorganisms genetically endowed with special characteristics to enhance their deadliness. To prevent theft of biological agents from our laboratories we may expect stricter security requirements. To ensure legitimate use of these agents we might expect stricter facility, equipment and procedural standards for our laboratories. Any future domestic bioterror incidents may lead to expanding the list of agents and more stringent controls on their possession, use and transfer.

Advances in understanding the basic biology of the cell as well as the genetic code and the manipulation of genetic information has spawned the new, but explosively growing, field of biotechnology. Biotechnology can be divided into those activities that are conducted in the laboratory and those activities that involve the introduction of biotechnological products into the environment. The first step in the development and testing of biotechnological products almost always takes place in the laboratory. Laboratory activities include growing cells, tissues and organs in vitro, the development of products for therapeutic use from these cells, the isolation and identification of genes from genetic material and the introduction of genes into microorganisms or other cells of plant, animal or human origins forming genetically modified organisms (GMOs). We can also include the laboratory aspects of transplantation of animal organs into humans in this category. The are many safety issues associated with protecting the laboratory worker from hazardous exposures such as hitchhiking contaminants in cultured cellular materials, genetic materials contained in transfer vectors, genes coding for virulence factors or toxins or, inadvertently creating hazardous agents by genetic transfers, and protecting the cellular products from contaminants, such as cultured cells or organs which may be used *in vivo* for transplantation. The diversity and complexity of this explosively growing area will provide many challenges and many opportunities for ABSA.

After being genetically engineered in the laboratory, the GMO's may be released into the environment for a variety of purposes. We are now familiar with potatoes and other vegetables and plants genetically engineered to make their own pesticides, strains of strawberries genetically engineered to survive freezing temperatures, and genetically engineered microbes that can "eat" oil or other materials. GMO's that are developed and tested successfully in the laboratory may prove a disaster when released into the environment. As a consequence environmental releases need to be carefully planned and monitored and risk assessments need
to be performed and approved by government representatives. The process must be carefully regulated from beginning to end. The increasing number of environmental releases of GMO’s is spawning a huge new discipline. In recognition of this several international meetings have been held that address “biological safety” in the environment. The American Type Culture Collection (ATCC) last spring proposed a meeting “Principles of Biological Safety and Risk Assessment” that dealt almost entirely with the environmental releases of biotechnological products and GMO’s. Environmental biosafety goes beyond our traditional roots in the laboratory and ABSA needs to decide if it has the interest and enthusiasm to bring this discipline under its umbrella.

Technological advances in communications, information management and transportation have made our world smaller. People can be transported around the world in a day. Huge amounts of information can be transmitted around the world via the Internet or cellular phones in a matter of minutes. We can expect more information to be available to our members and faster and wider communications via the Internet. We can expect our communications through our newsletter, journal, and training courses to become available electronically. The Journal of Emerging Diseases is available entirely through the Internet. Annual Conferences may be attended electronically via video conferencing and many training courses may be available electronically. Remote sensing of laboratory facilities and equipment is being installed in the newest high containment labs as is remote video surveillance for safety purposes. Perhaps one day laboratory inspection programs may be entirely automated and evaluated by electronic measures.

**Responding to the future**

I am concerned that there are a number of under developed areas within ABSA. One under developed area is associated with biological agents affecting livestock, poultry, and other animals. Some of the largest high containment facilities such as the Plum Island Animal Disease Center, and the newest, such as those at the Australian Animal Health Laboratories in Geelong and the New Canadian federal laboratories in Winnipeg work with infectious agents of livestock and other animals. Another under developed area is associated with biological agents affecting plants. Laboratory work with these agents also requires high containment laboratories aimed at preventing their escape to the outside environment. Both of these subject areas are dealing with emerging and reemerging diseases and harnessing biotechnology to produce more and better agricultural food products. Both subject areas require federal permits to work with selected animal or plant pathogens, and livestock and crops are excellent targets for biological warfare or terrorism. Other subject areas that are under developed include biological toxins, a number of which are now regulated under the select agent regulation (Federal Register, 1996), and insect vectors of infectious diseases of plants, animals and humans, which also require biological containment laboratories.

| TABLE 1 |
| Proposed Subject Matter Committees |
| Infectious agents of humans |
| Infectious agents of animals |
| Infectious agents of plants |
| Toxins from living organisms |
| Vectors of infectious diseases |
| Biotechnology in the laboratory |
| Biotechnology in the environment |
| Application of Information Systems to biological safety |

What can ABSA do to respond to strengthening under developed areas while also responding to the challenges of the future? One approach would be for ABSA to study the possibility of including in its organization “Subject Matter Specialties” or equivalents (divisions, committees, resources, etc.) (Knudsen, 1998). The American Industrial Hygiene Association contains subject matter committees and the American Society of Microbiology is divided into divisions. A proposed list of subject matter committees is shown in Table 1. Each subject matter specialty would be represented by a committee composed of a chair and members with deep interest and expertise in that specialty. The committee would serve as a technical resource for ABSA for answering questions, reviewing guidelines and regulations, and otherwise providing expertise in that subject matter area. The Specialty Committee would also be responsible for developing a session
in this specialty at the annual meeting. I believe that if we do not begin to focus on our specialty areas ABSA will remain rooted in the activities that we are performing today instead of growing to meet our future challenges.

I would like to express my deepest appreciation to the members of the American Biological Safety Association for bestowing upon me the honor of representing them as President this past year.

REFERENCES


Federal Register, 1976. Recombinant DNA Research Guidelines. 41:27902-27943


HEPA FILTER REPLACEMENT EXPERIENCE IN A BIOLOGICAL LABORATORY

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Australian Animal Health Laboratory, Geelong, Victoria, Australia

ABSTRACT

Integrity tests on 969 HEPA filters filtering air to and from rooms and laboratories have been done annually for 13 years. Filter replacements were analyzed by supply or exhaust air system, area serviced, primary or secondary situations and cause for replacement. The average annual filter replacement rate for installations filtering either supply or exhaust air, or vented gases were 2.9%, 1.9%, or 3.0% respectively. Initially, most failures occurred in the filter medium or the gasket seal. However, in recent years, there has been a general increase in filter reliability with filter blockage being the major reason for replacement. The replacement rates for exhaust filters in laboratory, animal room or other plant rooms were similar.

This study has shown that the critical biocontainment function of HEPA filters in this biological laboratory has been exceptionally reliable with little deterioration in performance over 13 years of service. Because a majority of filters (up to 78% for exhaust systems) have never been replaced, it was not possible to determine a meaningful life expectancy for filters.

INTRODUCTION

Historically, high efficiency particulate air (HEPA) filters were developed for the removal of radioactive particles from air streams in nuclear facilities. These filters have been used widely and are very effective also for the removal of infectious aerosols from air streams in medical, veterinary and other biological installations. The standards to which filters are manufactured and tested have been reviewed recently (First, 1996).

Where biocontainment is important, HEPA filters form a critical part of microbiological barriers. As air handling systems in buildings run continuously, the integrity and efficiency of HEPA filters must be checked at defined intervals to ensure biocontainment standards are met. In general, annual testing of filters is adopted to ensure the filter medium is integral, the filter is effectively sealed in its housing, and that the pressure drop across the filter is acceptable.

Reports concerning the reliability and performance characteristics of HEPA filters in nuclear (Carbaugh, 1982) and both nuclear and non-nuclear (Robinson et al., 1985) installations have been published, but there is no information concerning the longevity of filters in biological facilities (First, 1996). This paper reports the reliability of HEPA filters in air handling systems in a large veterinary laboratory over a 13-year period since commissioning.

MATERIALS AND METHODS

Laboratory Design
The high containment, animal health laboratory described was designed and built following the box-within-a-box principle. Within the outer microbiological barrier are three large suites of laboratories, having floor areas ranging from 960 to 1376 m² each having their own microbiological barriers. Each suite contains laboratories, small animal rooms and higher containment laboratories for more hazardous work. Separate again is a suite of 28 animal rooms for large animals and a necropsy room. Surrounding these core work areas are support service areas including glasswash, laundry, electronics and engineering workshops, storage, staff facilities, engineering plant rooms for both air handling and sewage treatment as well as an extensive corridor network.

Air Handling
The whole complex of laboratories, animal rooms and support services receives 100% fresh air drawn from outside the building into a common plenum, and particulate matter removed by a Vokes Trivee DG20 Type 1 filter tested to Australian Standard AS1132 (1973), having an average efficiency of 97% for No. 2 dust at a flow rate of 944 L/sec. Air
may be heated or cooled (but not humidified) prior to passing through another Vokes filter to one of the 45 air handling systems supplying air to various zones within the microbiologically secure area. A roughing filter pad (Email, Type 1, tested to Australian Standard AS1324.2 [1996], having an efficiency of 58% for No. 2 dust at a flow rate of 700 Lsec\(^{-1}\)) removes particles created by electric motors and belt-driven fans before the air finally passes through a 610 x 610 x 292 mm HEPA filter to its destination. For the 28 large animal rooms (that act as primary microbiological containment zones), necropsy and several isolation rooms, two HEPA filters are used in series for the supply air as a final safeguard against infectious particle backflow from contaminated rooms.

In large plant rooms, both supply and exhaust air are handled by banks of up to 28 filters in parallel. If the airflow through such banks falls below required levels (measured by a pressure drop >500Pa) because of medium blockages, it is likely that all filters in the bank need replacement.

Air is exhausted from laboratory and other areas through preliminary clarification prefilters having an average efficiency of 83% for No. 4 dust at a flow rate of 540 Lsec\(^{-1}\) prior to filtration through two 610 mm square, HEPA filters in series to ensure optimal removal of infectious aerosols. In animal rooms, both dander and roughing prefilters are included before the air leaves the room.

**Filter Installations**

All HEPA filters, whether supply or exhaust air, are mounted in separate, horizontal, cylindrical canisters with removable ends allowing direct access to both sides of the filter for examination and scan testing (Figure 1). The 610 mm square filters are sealed in mountings with compressible gaskets that meet required standards for airtightness measured by cold dioctyl phthalate (DOP) particle penetration. At the completion of testing, the canisters are pressure tested to confirm an integral seal of the end lids and the valves. Where two HEPA filters are installed in series, both filters are integrity tested sequentially.

**FIGURE 1**

Canisters with removable ends allowing easy servicing and scan testing of 610 mm HEPA filters.
In addition to supply and exhaust air systems, there are eighty-six 304 x 304 x 149 mm HEPA filters (mostly installed as pairs in series in vent lines) venting gases from effluent drains and collection vessels into engineering plant rooms. As these filters are usually exposed to air at higher relative humidities than the filters in supply and exhaust systems, the canister housings are routinely held at 50°C to minimize water condensation on the filters.

The same smaller HEPA filters are used in 55 local ventilation systems to filter air from seldom-used shower cubicles which cross architectural or microbiological barriers between the plant rooms or emergency exits. The 304 mm square filters are mounted in cylindrical canisters and integrity tested annually in the same way as the larger filters in air handling systems.

Filters

When the laboratory was constructed and commissioned in the years prior to 1985, excess HEPA filters were purchased from either Email Airhandling, Australia (now BTR Environmental Pty. Ltd.) or Gelman Sciences (now Clyde Apac, Australia) companies. A contemporary definition for such HEPA filters has been published recently (First, 1996) and all filters in this study conform with this description. Initially, all were acceptance tested on-site before installation using the British Standard Sodium Flame Test BS 3928 (1969) to ensure compliance with supply contract specifications. Surplus filters have been stored on-site ever since and used as replacements.

All installed filters are tested annually (or biennially) to meet integrity specifications and are replaced as soon as the acceptable limits are exceeded. There are no predetermined replacement periods for filters. Because of the unique and patented canister design, complete and convenient scanning can be done for “pinhole” leaks in the medium, gasket or mounting plate for every filter and canister, allowing all defects of consequence to be detected. This mounting design allowed comprehensive scan testing to Australian Standard AS 1807.6 (1989) and the opportunity for a defective filter to be discarded as soon as a local fault causing non-compliance was detected.

During testing, relevant data was recorded and considered. Minor defects noted during previous tests could be checked thoroughly for any change. When pinhole leaks were detected in an otherwise sound filter, the filter was removed and the hole repaired with a silicone elastomeric sealant. After retesting for performance, such filters were available for re-installation in supply air canisters only.

During the years 1994-1997, 20 exhaust air HEPA filters were replaced by a similarly-sized but higher efficiency ultra low penetration air-filters (Flanders Filters, Inc., USA) for experimental purposes (Jarmiska, Martin, and Morawska, 1997).

RESULTS

Replacement Data for All Filters

Initially, all air supply, air exhaust, vent or local area filters were checked annually for integrity as described in Methods and Materials. Since 1994, some supply and exhaust filters in areas having lower microbiological hazards have been tested only every second year.

Data on the total number of filter replacements has been recorded since 1985 and is shown in Figure 2. The data is divided to show the three types of filter systems—supply air, exhaust air, and sewage vent/local filters. Average annual replacement rates for these three filter systems were 2.9%, 1.9% and 3.0% respectively (Tables 1-3).

Replacement Data for Supply Air Filters

Reasons for replacement of filters at this laboratory have been ascribed to four groups: failure of the filter medium, failure of the gasket seal between the filter and mounting plate, insufficient air flow through the filter, and other miscellaneous problems.

Table 1 shows a breakdown of replacement reasons for the 317 supply air filters. Medium and gasket failures occurred infrequently after the first few years of installation. However, blockages of filters began to occur more frequently after 1990. In total, 121 (38.1%) were replaced for all reasons over the 13-year period.

Replacement Data for Exhaust Air Filters

Table 2 shows a breakdown of replacement reasons for the 511 exhaust air filters. Failure and blockage of the filter medium were the most common reasons for filter replacement and these values
FIGURE 2
Annual HEPA filter replacement percentages. Total filter installations were 317 filters in supply, 511 in exhaust, and 141 in vent and local systems.

TABLE 1
Summary of Supply Air Filter Replacements
Of 317 supply air filters installed, 121 (38.1%) have been replaced for all reasons.

<table>
<thead>
<tr>
<th>YEAR</th>
<th>MEDIUM FAILURE</th>
<th>GASKET FAILURE</th>
<th>BLOCKAGE</th>
<th>DAMAGE / OTHER</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1985</td>
<td>7</td>
<td>4</td>
<td></td>
<td></td>
<td>11</td>
</tr>
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<td>15</td>
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<td>1997</td>
<td></td>
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<td>16</td>
<td>18</td>
</tr>
<tr>
<td>TOTAL</td>
<td>16</td>
<td>14</td>
<td>90</td>
<td>1</td>
<td>121</td>
</tr>
</tbody>
</table>
### TABLE 2
Summary of Exhaust Air Filter Replacements
Of 511 operating exhaust air filters, 125 (24.4%) have been replaced for all reasons. Numbers in brackets represent primary filter replacements.

<table>
<thead>
<tr>
<th></th>
<th>MEDIUM FAILURE</th>
<th>GASKET FAILURE</th>
<th>BLOCKAGE</th>
<th>DAMAGE / OTHER</th>
<th>TOTAL</th>
</tr>
</thead>
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<tr>
<td>1985</td>
<td>14 (10)</td>
<td>3 (2)</td>
<td>3 (1)</td>
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<tr>
<td>1986</td>
<td>7</td>
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<td>6 (6)</td>
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<td>15</td>
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<tr>
<td>1987</td>
<td>7 (3)</td>
<td>2 (2)</td>
<td>1 (1)</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>1988</td>
<td>11 (1)</td>
<td></td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>1989</td>
<td>4 (3)</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>1990</td>
<td>6 (1)</td>
<td>1</td>
<td></td>
<td>2 (1)</td>
<td>9</td>
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<tr>
<td>1991</td>
<td>1 (1)</td>
<td>17 (17)</td>
<td>2 (1)</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>1992</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1993</td>
<td></td>
<td></td>
<td>1 (1)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1994</td>
<td>1</td>
<td></td>
<td>1</td>
<td>6 a (1)</td>
<td>8</td>
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<tr>
<td>1995</td>
<td>1 (1)</td>
<td></td>
<td></td>
<td>3 a</td>
<td>4</td>
</tr>
<tr>
<td>1996</td>
<td></td>
<td></td>
<td></td>
<td>10 a (1)</td>
<td>10</td>
</tr>
<tr>
<td>1997</td>
<td>1 b</td>
<td></td>
<td>1 (1)</td>
<td>2 a (1)</td>
<td>4</td>
</tr>
<tr>
<td>TOTAL</td>
<td>54 (19)</td>
<td>9 (5)</td>
<td>27 (26)</td>
<td>35 (6)</td>
<td>125</td>
</tr>
</tbody>
</table>

\(a\) Optional replacements to test more efficient filters.

\(b\) This failure occurred in one of the replacement filters (noted in a above) and not a HFPA filter.

### FIGURE 3
Annual HEPA exhaust air filter replacement percentages. Total filter installations were 160 in general laboratories, 125 in large animal rooms, and 226 in support areas.
were higher in the first four years after installation. The “damage/other” column included some mechanical damage done to filters while testing methods of biological assessment of the decontamination procedure (Abraham et al., 1996), and 18 optional replacements done (from 1994 to 1996) to test a different type of higher efficiency filter. Of 511 exhaust filters, 125 (24.4%) were replaced for all reasons over the 13-year analysis period.

The figures in brackets in Table 2 represent those filters replaced in the primary position of each pair in series. Of 125 replacements, 56 (45%) were in primary filters.

Analysis of Exhaust Air Filter Replacements by Zone

Of the 511 exhaust filters, 160 were in regular laboratories and small animal rooms, 125 in large animal rooms, and 226 in support areas and plant rooms. Figure 3 shows a breakdown of filter replacements in these three zones, the average annual replacement rates being 1.7%, 2.7%, and 1.5% respectively.

Analysis of Replacements of Vent and Local Filters

Table 3 shows a breakdown of reasons for all replacements in filters venting sewage collection pipes and local areas such as staff showers. All of these filters are 304 mm square filters (compared with 610 mm square filters in air supply/exhaust systems) and are considered together for this reason. Of 141 such filters, gasket failure was the most frequent reason for replacement. Vent filter canisters are held routinely at 50°C to minimize the possibility of water vapor condensing on the filters in these low air flow systems. On average, 3.0% of vent and local filters required replacement annually.

DISCUSSION

This report describes the reasons for HEPA filter replacements in in situ air handling systems in a large microbiological laboratory over a 13-year period. As previous authors (Carbaugh, 1982; Robinson et al., 1985) have reported on the life expectancy and aging of similar air system filters in nuclear energy plants, this study provides compara-

### TABLE 3
Summary of All Vent and Local Filter Replacements

<table>
<thead>
<tr>
<th>Filter Year</th>
<th>Medium Failure</th>
<th>Gasket Failure</th>
<th>Blockage</th>
<th>Damage / Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1985</td>
<td>1</td>
<td>2</td>
<td></td>
<td>3</td>
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<td>1986</td>
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<tr>
<td>1997</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td>14</td>
<td>24</td>
<td></td>
<td>18</td>
<td>56</td>
</tr>
</tbody>
</table>
tive data for filters acting as microbiological biocontainment devices in air handling systems. No consideration of biosafety cabinet filters was included in this study.

Until 1994, all filters in this study were checked annually for integrity as described in Methods and Materials. Since then, the testing of a minor proportion of supply and exhaust filters servicing lower microbiological risk zones was extended to two yearly intervals. This change is not considered to have altered significantly the data presented here.

For all filter types, the three main reasons for filter replacements since 1985 have been blockage of the filter medium, defects (usually “pinhole” leaks) in the filter medium and defects in the gasket seal securing the filter frame in its housing. Unexpected reasons for damage caused by handling accidents, smoke exposure and steam condensation were irregular and minor overall causes for filter replacement (Tables 2 and 3). Medium and gasket problems occurred mainly during the first five years of installation; there have been relatively few such faults during the past eight years (Tables 1-3). Of filters in 969 installations included in this survey, 302 (31%) have been changed for all reasons during 13 years. This corresponds to an annual replacement rate of 2.4% which is significantly lower than published values for U.S. Department of Energy nuclear plant sites (Carbaugh, 1982) where the annual average replacement rate over a three-year period was >19%. Carbaugh reported that 12% of all filters actually failed during this period because of ruptures in the filter medium, faults in the filter frame or defects in the filter seals. In contrast, the total number of actual failures for these reasons in the present analysis was 131, or an annual average of 1.0% over the 13-year period. No filters, even those accidentally wetted by steam leaks, have ever failed catastrophically at this facility. The largest point penetration in the filter medium detected during the past six years by the cold DOP method was 0.9%, and only six point penetrations have exceeded 0.5%.

Because of their critical function in minimizing the possibilities for disease escape via infectious aerosols, data relating to exhaust air filters was examined more closely. During the past seven years, the filter replacement rate has fallen to 0.1% for exhaust filters (compared with 0.2% for all filters), indicating an increasing reliability of this part of the biocontainment barrier; inherently defective or less robust filters having been “weeded out.” The data was also studied to see if filters in particular exhaust canisters were replaced more frequently. Of the 125 replacements shown in Table 2, 110 were replaced once, 13 replaced twice and two replaced thrice. It follows that 401 (78.4%) of filters in the exhaust system have never been replaced. Calculations were attempted to determine the mean life expectancy for such filters. However, since more than half of the filters have not yet been replaced and their reliability is actually increasing (Table 2), the value derived had little practical meaning as it appeared to exceed the 50-year predicted life expectancy of the biocontainment laboratory (calculations not shown).

Carbaugh (1982) reported filters in nuclear industry facilities were changed five times more frequently for blockage than for other reasons. This contrasts with the present study where over the 13-year period, there were 117 replacements caused by blockages compared with 185 for all other reasons. Although some supply and exhaust filters in areas having lower microbiological hazards have only been tested every second year since 1994, we do not believe this variation has skewed the overall results as, apart from blockages in some supply filters, there have been relatively few filter replacements over this period.

The present data shows that HEPA filters are lasting significantly longer at this laboratory than has been reported elsewhere. There are a number of reasons that may contribute to, or be the cause of this effect. In general, data may be distorted for a number of reasons, but in this study, many of these can be excluded: 1) comprehensive records have been carefully kept since 1985, 2) criteria for filter replacement are stringent (see Methods and Materials) and conform with contemporary international standards, 3) annual filter testing has been completed on time for the majority of the period, 4) complete manual scan testing for each filter has been possible because of unique canister design allowing all pinholes of consequence to be detected, and 5) comprehensive staff training programs and adherence to strict quality assurance standards ensuring filter replacements were made at the earliest time faults were identified.
A number of factors can be suggested that have contributed positively to the relatively long filter life observed: 1) the unique design of each filter in its own cylindrical canister (Figure 1) has minimized filter handling and the possibilities for damage during decontamination and scan testing, 2) the initial filters were purchased following a well-researched analysis resulting in a tender specification document requiring only high quality materials and construction techniques, 3) the use of a laminated timber frame provided a more rigid structure than alternative metal frames, perhaps resulting in less distortion during installation and handling, 4) the method of securing the filters to the rigid canister mounting plate provided a more even load distribution on the filter frame than many commercially-available filter mounting frames thus contributing to the excellent performance achieved to date, 5) the filtration of all supply air through at least one HEPA filter reduces the particle impact on exhaust filters, 6) thorough efficiency and integrity testing of all filters prior to installation meant the initial quality of filters installed was high, 7) where two filters are installed in series, the secondary filter is somewhat protected from particles by its primary counterpart, and 8) since all filter housings are within the microbiological barrier, the filters have been held at a relatively constant temperature of 21-24°C (or 50°C for the 86 filters venting gases from sewage treatment pipes and vessels).

Water, dust and smoke led to premature deterioration of filters on occasions. Examples of these effects can be seen as fluctuations of replacement rates where condensation of water vapor in vent filters in 1987 caused permanent damage to eight filters (Table 3), and in 1991 to 17 filters following the release of smoke from a faulty incinerator (Table 3). In 1993, a large bank of 28 filters providing filtered air to a large plant room needed to be replaced because of an increased pressure drop across the filters caused by the blocking effects of dust particles (Table 1).

Most of the zones in the laboratory were relatively clean and dry in terms of air quality. Exceptions were engineering plant rooms where machinery and fans released particles to the surrounding environment. Relatively few chemicals that might cause filter damage were released from any area. This contrasts with the observations of Carbaugh (1982) who noted that the highest frequency of filter change-outs occurred in environments having higher concentrations of hydrofluoric acid and other fluoride-containing gases. The only potentially damaging chemical consistently exposed to filters in the present report has been formaldehyde (Abraham, et al., 1996) and it is reasonably clear that it has had little effect on the aging of filters.

Animal rooms are zones where animal dander particles and possibly gases such as ammonia were considered likely to cause an increase in filter replacement rates. This effect is not substantial as the average annual replacement rate for animal rooms of 2.7% compares favorably with replacement rates of 1.7% in laboratories and 1.5% in support areas (Figure 2).

One of the types of fault leading to medium failure is a pinhole leak, or tiny holes where local concentrations of the test chemical, diocetyl phthalate, exceed the value permitted for an intact filter. Annual records for individual filters have allowed small pinhole leaks to be identified and then followed in subsequent years where the filter performance was still within the prescribed limits. The conclusion drawn by the authors (data not shown) is that small pinhole leaks do not get larger with time. It was also surprising to see that pin hole leaks and other medium defects do not seem to be caused by particle impact (Table 2) as the frequencies of primary and secondary filter replacements in exhaust systems were not significantly different. In such circumstances, the secondary filters would have been free from significant particle impact damage.

It is important to note that all filters used in this laboratory were of a similar age (14-16 years) as excess filters were purchased prior to 1984, tested and kept in storage. This reserve of filters has not yet been expended. The replacement rate for filters during this study has decreased with time (Figure 2). When considering failure of the filter medium and gaskets (Tables 1, 2, and 3), it became clear that aging has not yet led to a significant increase in defects in filter integrity. In this respect, this laboratory does not have predetermined times for filter replacement as a preventative maintenance measure. In addition, no catastrophic failure of filter medium has been observed during the whole of this study. This information provides some reassurance for the concerns of First (1996) who suggests that
there is a general lack of data concerning the longevity of HEPA filters in biological applications, and that there may be merit in replacing filters after some pre-determined "useful life" span. As far as HEPA filters in air handling ducts are concerned, this study shows >69% of filters installed in 1985 are still functional and integral, a period significantly longer than the nominal five years suggested by First (1996) for biosafety cabinet filters.

Another aspect of filter aging is a possible decrease in the tensile strength of the fibers in the filter medium. Such analyses have commenced at this laboratory and preliminary data suggests that after 14 or more years, either in service or in storage, the media of representative filters still conformed with the relevant specification (MIL-STD F-51079) even though there is some loss of mechanical strength in the medium (S. Edwards, personal communication). Results of this work are to be published soon, but appear to be inconsistent with the reports of Robinson, et al., (1985) who observed filter deterioration in respect to paper strength and aluminum spacers in HEPA filters in both nuclear and non-nuclear facilities.

Filters were originally purchased from two manufacturers, Email and Gelman. The different filters were installed in the facility randomly. The available evidence during this study showed that the replacement rates for both types of filter were similar (data not shown), but exact figures were not available because when filters were replaced over the years, there was no specific policy to replace a particular brand of filter with a new one from the same manufacturer.

For economic reasons, filters with pinhole leaks failing to meet performance criteria were patched with silicon elastomer, tested and a few filters returned to service in specific, supply air situations associated with low microbiological hazards. Subsequent performance of such repaired filters was comparable to that of intact filters (data not shown).

Filters of two sizes were examined in this study. Data related to larger 610 mm square filters that filter supply and exhaust air is shown in Tables 1 and 2 while data related to smaller 304 mm square filters that service local zones and sewage vent lines is shown in Table 3. The annual, average replacement rates for the larger and smaller filters were 2.2% and 3.0% respectively. Although the filtration functions were different, their life spans were comparable indicating that major differences between the two sized filters probably do not occur. It is clear from Figure 3 that blockage has not been a problem with filters in local and vent systems and this is no doubt related to the lower airflow through and particle load on these filters.

Of the 141 filters included in the data in Table 3, 86 were servicing sewage vent lines. These filters were thus usually exposed to higher relative humidities than the filters in supply and exhaust systems. To minimize the possibilities of water condensation on the filters, the canister housings were routinely held at 50°C. Consequently, these filters were exposed to a more humid and warmer environment leading to a higher failure rate of the gasket seals (Table 3), relative to their medium failure, blockage or other problems.

ACKNOWLEDGEMENTS

We gratefully acknowledge Bob McGough, Neil Slater, Garry Gould, Jenny Martin, Tony Freeman, and Rob Dandy for their diligent assistance with filter testing. Steven Edwards, Gary Smith, Albert Trajstman, and Vivienne Lewis provided valuable comments on the manuscript.

REFERENCES


RISKS ASSOCIATED WITH LIQUID NITROGEN CRYOGENIC STORAGE SYSTEMS

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ABSTRACT

Laboratory staff should be aware of the potential for personnel exposure and cross-contamination of improperly stored samples in liquid nitrogen cryogenic storage systems. Published reports indicate that two viruses, Hepatitis B and Vesicular Stomatitis Virus, retain infectivity after suspension in liquid nitrogen. This information confirms the standard biosafety recommendations on the use of personal protective equipment when adding or removing samples from a cryogenic storage system, and on the need to decontaminate equipment when it is being decommissioned or repaired.

Hepatitis B Virus Transmissions

A literature search revealed no laboratory-acquired infections attributed to cryogenic storage systems. However, there is a published report that Hepatitis B Virus (HBV) retained infectivity for over two years when suspended in liquid nitrogen.(5) In one British hospital, there were six cases of acute HBV infection reported to infection control among multiply transfused patients undergoing cytotoxic treatment. All six patients were seronegative for HBV when treatment was initiated. A study of the transfusion history of the patients revealed no common donor. Other potential common links among the cases were evaluated and eliminated. The times the patients were actually admitted to the hospital did not overlap, (one patient had been treated in a different clinic); and the patients had no personal contact with each other. Even the healthcare workers who cared for the patients were ruled out as a common factor. Only one common link was identified—the harvested bone marrow or peripheral stem cells were stored in the same cryogenic tank.

Cytotoxic treatment involves administration of toxic doses of chemotherapeutic drugs, with the patients' own bone marrow or peripheral-blood stem cells being stored for possible re-infusion. Since a high rate of cell viability on thawing is critical, the standard for this type of cell storage is a heat-sealed bag of blood cells stored in the liquid phase of cell storage tank.

A newly commissioned tank was used to store the source patient’s blood; subsequently, over a two-year period, the bags from the other five patients were added to this tank. It was known that a bag of the source patients’ blood had leaked during cryogenic storage. To investigate the potential for HBV transmission, the authors allowed the suspect tank to thaw and analyzed the resulting aqueous liquid. Human DNA sequences and Hepatitis B surface antigen (1 mg/ml) were detected. Polymerase chain reaction studies indicated that the residue from the tank contained Hepatitis B sequences identical to that of the four patients’ blood samples available for comparison. (No samples from patient #5 were available for study).

But how was HBV transmitted from the source patient to the other patients? The two remaining bags of the source patient’s blood, which appeared to be intact, were removed from liquid nitrogen storage. The bags split open as they thawed; apparently, liquid nitrogen had penetrated the heat seals of the bags. The authors hypothesize that the HBV contaminated the other bags during storage and then was introduced to the patients during re-infusion. Since the type of blood bag used was defective, the ports on the bags could have become contaminated, or the contents of the bag may have come into direct contact with the liquid nitrogen.

This particular type of cryogenic storage bag was recalled by a HAZARD WARNING issued by the UK Medical Services directorate, and this product is no longer used for clinical cryogenic storage. The critical point for biosafety professionals, which has not been previously documented, is that Hepatitis B virus which had leaked directly into liquid nitrogen retained infectivity for a two-year period.

Vesicular Stomatitis Virus Survival

Schering scientists discovered that a glass vial of
Vesicular Stomatitis Virus (VSV) had shattered during liquid nitrogen storage. Since a literature search at that time did not indicate whether the VSV could potentially retain infectivity after suspension in liquid nitrogen, an experiment was conducted. Liquid nitrogen aliquots were aseptically removed from the storage tank and were allowed to evaporate in their container in a biosafety cabinet. Small volumes of saline were used to wash the containers; VSV was detected in the washes. To confirm the finding, VSV was added directly to volumes of liquid nitrogen; there was no loss of infectivity.

**Implications for Biosafety Professionals**

Review of storage conditions where biohazardous samples are maintained in liquid nitrogen is essential to a biosafety audit. More research is required to define the classes of viruses and/or other microorganisms that retain infectivity when inadvertently suspended in liquid nitrogen. The data available on two viruses may help to educate staff on the need for careful use of liquid nitrogen cryogenic storage systems. Proper personal protective equipment when adding or removing samples from such systems is required. An understanding of the limitations of the type of cryovial used minimizes the potential for contamination of the liquid nitrogen.

An excellent resource on the mechanics of liquid nitrogen cryogenic storage is the *Cryopreservation Manual* distributed by NALGE Nunc International Corp. Despite such publications, researchers often fail to follow manufacturer recommendations. The most common container for cryogenic storage is the plastic cryovial. These are supplied in packages marked “FOR VAPOR STORAGE ONLY”, however, this warning is often ignored. Using the vial in the liquid phase of the tank increases the risk of exploding vials and cross-contamination of samples. Rather than paraphrase information related to the safety considerations associated with cryogenic storage, they are reproduced from the NALGENE® *Cryopreservation Manual* below.

“Safety precautions must be observed throughout the preservation and maintenance process. All work with hazardous cultures should be performed under proper containment, and U.S. Public Health Service Biosafety guidelines should be adhered to at all times.

Animal cells may contain adventitious viral agents that require special handling, and all animal cells that have not been thoroughly characterized should be handled at Biosafety Level II. At this level, laboratory staff must have training in handling pathogenic agents and work under the direction of a competent scientist. Access to the laboratory must be limited and biological safety cabinets must be used for large-volume work or when aerosols are generated.

Low-temperature storage of cells presents unique hazards that necessitate safety precautions. Cryogenic temperatures can result in exposure of personnel to extremely cold conditions, and precautions must be taken to protect personnel during operations in liquid nitrogen freezers. Insulated gloves and long-sleeved laboratory coats or other garb protect the skin from exposure. It is extremely important to wear a full-face shield and neck shield when working in the liquid portion of a liquid nitrogen freezer. As noted above, improperly sealed glass ampoules may explode when retrieved from liquid nitrogen. To minimize the risk of potential explosions, place the ampoule or vial in the vapor phase for 24 hours. A face shield that provides neck protection should be mandatory when retrieving vials from liquid nitrogen. The use of NUNC™ CryoFlex™ is also strongly recommended. See warning.

**WARNING:**

Do not use vials for storage in the liquid phase of liquid nitrogen unless correctly sealed in NUNC™ CryoFlex™ Tubing (Cat. No. 343958). Improper use may cause entrapment of liquefied nitrogen inside the vial and lead to pressure build-up, resulting in possible explosion or biohazard release. Use appropriate safety procedures as outlined in this manual when handling and disposing of vials.

Special precautions must always be taken when working with hazardous biological materials at liquid nitrogen temperatures.
Always thaw and open vials containing hazardous material inside a biological safety cabinet. Be prepared for exploding and leaking ampoules/vials. Broken ampoules in a liquid nitrogen freezer are a potential source of contamination (3), and contaminants may survive, despite the extremely cold temperatures. When a liquid nitrogen freezer becomes contaminated, the entire unit should be decontaminated after warming to room temperature. When closing down a liquid nitrogen freezer that is not obviously contaminated, remove all material to be retained, warm the unit to room temperature and disinfect it prior to further handling.”(4)*

Limitations of Liquid Nitrogen Storage Systems

The temperature at which frozen materials are stored affects cell viability. The lower the temperature, the longer the cells can be recovered. Ultimate stability of mammalian cells cannot be assured unless the cells are maintained below -130°C Centigrade (C). Liquid nitrogen units that provide all-vapor storage are ideal, but the working temperature at the top of the unit must be maintained below -130°C. This requires careful adjustment of the liquid level. The Cryopreservation Manual states that this can be achieved in most working units, “however, the design of some models means the amount of liquid nitrogen necessary to attain the proper working temperature will reduce the amount of usable storage space.”(4) Some researchers may feel that raising the level of liquid nitrogen will improve viability because the cells will be kept at a lower temperature; it is important to explain that storing vials designed for vapor storage in the liquid phase may contaminate the liquid nitrogen and cross-contaminate stored samples.

One manufacturer provides a plastic sleeve for cryovials stored on a wand; encased in this sleeve; the cryovials may then be submerged in the liquid phase. For cryovials stored in boxes, some researchers leave the bottom spaces of their storage rack empty. This method is satisfactory only as long the level of liquid nitrogen does not exceed the level at which samples are stored. Two problems with this method are that: 1) adequate control of the liquid level may be difficult in manual-fill systems and 2) maintaining this system requires the cooperation of the users. A better option for biohazardous sample storage is an automated-fill liquid nitrogen system with a platform between the liquid and vapor phase. The vapor platform is offered by manufacturers and relatively inexpensive. Shorter racks are purchased to sit on top of the vapor platform. After setting the liquid nitrogen level to just below the platform, the system controls can be locked to prevent tampering. This measure will provide the maximum achievable control of a liquid nitrogen storage system and, given the potential for viral contamination, may be the best solution for biohazardous sample storage.

The standard operating procedure (SOP) for a cryogenic storage unit should include information on the decontamination procedures to be followed before service or decommissioning. The SOP should include informing the Laboratory Director if problems occur such as overfilling of the tank beyond the required level or dropping of vials into the liquid phase of the cell storage system.

Mechanical Freezer Systems

New developments in ultralow freezer technology may allow mechanical freezers to replace liquid nitrogen for cryogenic storage in research and clinical applications.(2) The new compressor technology provides air-phase storage at -140 and -150°C without the need for liquid nitrogen. According to the manufacturer, mechanical freezer systems will actually be less expensive than liquid nitrogen cell storage systems when one factors in costs associated with the rental and delivery of liquid nitrogen tanks.(2) This is an exciting new development. However, for most research programs, it will be years before replacement is considered. Until new mechanical freezer technology replaces liquid nitrogen storage systems, biosafety professionals must continue to evaluate standard operating procedures for the use of the numerous liquid nitrogen storage tanks in laboratories. This article was submitted to raise awareness of the biohazard potential and usage limitations of cryogenic storage systems.

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REFERENCES


Simione, F. 1998. American Type Culture Collection in cooperation with NALGE Nunc International Corp. CRYOPRESERVATION MANUAL.


U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institutes of Health, Bethesda, Maryland. 1988. Biosafety in Microbiological and Biomedical Laboratories.
ARTICLES

Abraham, G.; Smith, P.M.L.; & Nguyen, S.
The Effectiveness of Gaseous Formaldehyde Decontamination Assessed by Biological Monitoring
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Abraham, G.; Hooper, P.; Williamson, M.; Muschialli, J.; Martin, D.; Duff, I.; & Edwards, S.
Handling of Large Experimental Animals Infected with a Risk Group 4 Virus
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Autoclave Emissions—Hazardous or Not
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Johnson; B.; Winters, D.R.; Shreeve, T.R.; & Coffey, C.C.
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