Method Detection Limits and Non-Detects in the World of Microbiology

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Abstract
Examining indoor air for microorganisms is generally performed by sampling for viable microbes, growing them on sterile media under ideal conditions, and counting the colony forming units (CFUs). A negative result does not indicate that the source of the sample was free of fungi or bacteria, however, only that if present, the number of viable fungi or bacteria was below the limits of detection of the test.

This situation is problematic where government officials declare a building inhabitable on the basis of "no growth in any environmental sample". The Government Accountability Office (GAO) and the public want the answer to a more pragmatic question: "Is this building contaminated?" Likewise lack of confidence in negative results is at issue where professional remediators of water damaged buildings are trying to gauge efficacy of their efforts to eradicate fungi and their spores, or where in-duct ultraviolet light technologies are being tested for kill of vegetative bacteria and the manufacturers wish to publish efficiency ratings for the devices.

The dichotomy is that decision makers and building occupiers want to know with certainty whether dangerous or potentially dangerous microbiological organisms still exist in the structure while even the most sophisticated sampling and analysis methods available cannot provide conclusions with 100% certainty. Enter here government risk assessors who must grapple with the problem using the same data.

This paper explores sampling indoor air and surfaces for microorganisms, their analysis by conventional and state of the art methods, the interpretation of the results, and the state of governmental regulation of acceptable levels of such organisms and their effluents.

Introduction

The U.S. Environmental Protection Agency (EPA) defines the method detection limit (MDL) as the lowest amount that differentiates a sample that contains the substance from one that does not, and the quantification limit as the lowest amount of a substance that can be measured with a stated level of confidence (40 CFR 1984). In chemistry, it is understood that non-detects may contain some of the analyzed substance but in a small enough quantity that it can be considered zero and of little concern. Government risk assessors evaluating risk of highly toxic substances may require the development of more sensitive sampling and analytical methods.

The traditional thought processes regarding MDLs are strained, however, when measuring microscopic organisms such as vegetative bacteria, fungi, spores, and viruses. Traditional methods for analyzing microbes are so sensitive that they can detect even one viable entity, that is, one capable of reproducing under ideal growth conditions for the organism, but several issues remain. Air samplers, for instance, have to be sited correctly to capture any viable organisms. Sampling methods can kill organisms via desiccation or otherwise fragment them and render them incapable of growth and therefore detection by viability methods, yet still exist in the environment as dangerous allergens. Analysis methods are based on morphological characteristics and can be inaccurate. Fragments and products of microorganisms, such as mycotoxins, must be sampled and identified another way. Any sampling and analysis plan must provide for both viable and non-viable microbes to be representative of what is really there.

Three Recent Scenarios That Illustrate the Problem

Determining residual biological threat agent following cleanup from a terrorist attack
In September and October 2001, letters containing the spores of *Bacillus anthracis* (anthrax) sent through the U.S. mail were opened by the staff in the offices of two U.S. senators and members of the media. A total of 22 persons contracted anthrax disease and five died. The U.S. Postal Service (USPS), the Centers for Disease Control and Prevention (CDC), and the EPA performed several sample collection and analytical activities in postal facilities in 2001 for the purpose of detecting anthrax. The sampling strategy was to target the most likely areas where anthrax might be found. On April 5, 2005, 3 ½ years later, in its testimony before the House of Representatives Subcommittee on National Security, Emerging Threats, and International Relations, the U.S. Government Accountability Office (GAO 2005) stated that probability sampling would have been a better choice. The GAO believes that probability sampling would have allowed decision makers to determine whether a building is contaminated with some defined level of confidence *even when all results are negative*. GAO wants validated sampling. Even now, 4 ½ years after the bioterrorist incident, sampling and analysis strategies are still at issue.

Determining residual fungi from cleanup after a natural disaster
On August 29, 2005, hurricane Katrina brought category 4 force winds and flood damage to several hundred square miles of the Louisiana and Mississippi Gulf Coast. Several levees were breached flooding up to 80% of the city of New Orleans and large areas of surrounding parishes. The U.S. Army Corp of Engineers declared the flooded areas "unwatered" on October 11, 2005, but the aftermath of soaked buildings and furnishings has brought a legacy of mold proliferation on a scale rarely seen in U.S. history. For those homes that are deemed remediable, a huge cleanup effort is being undertaken. Little of this work can be performed by the residential owners themselves because the microbial contamination is so extensive. For surface areas of 10 square feet or more affected by mold, EPA recommends professional cleanup. No sampling is recommended for areas with visual mold growth, but during the cleanup professional remediators must perform to standards that bring the property to a condition at least as good as existed before the flood. Any such assurance will involve post-remedial sampling for residuals from fungi. What sampling and analysis plan will provide such confidence?

Determining residual bioaerosols after treatment with an efficient technology
Manufacturers are interested in verifying the airborne inactivation efficiency of their in-duct ultraviolet (UV) light air cleaning systems on the culturable challenge bioaerosol in the air circulating in heating, ventilation and air conditioning (HVAC) systems. In a test based on the inert particulate testing method for filters that is the basis for the American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE 1999) Standard 52.2, the testing organization tests a full-scale module of each UV device by installing it in a standard full-scale test duct that meets the ASHRAE standard. The selected challenge aerosol is injected into the inlet air stream upstream of a mixing baffle. Bioaerosol concentration is measured both downstream and upstream of the device to obtain the ratio of the surviving concentration to the challenge concentration of viable test organisms. Challenges with spore-forming bacteria such as *Bacillus atrophaeus* or a virus such as MS2 provide calculable efficiencies since they have measurable downstream survivors. Vegetative bacteria such as *Serratia marcescens*, however, are easy to kill with UV light and therefore often have *no measurable survivors downstream of the device*. How should efficiency for such bioaerosols be reported?

Solutions: State of the Science of Decontamination after a Terrorist Attack

At an EPA National Homeland Security Research Center Workshop (Dun et al 2005), Kenneth Martinez of the Centers for Disease Control (CDC) stated that the purposes for environmental sampling after a terrorist attack include assessing the nature and extent of contamination, identifying the sources, supporting risk assessment and public health decisions, and guiding re-occupancy decisions. The three sampling stages of a response are screening, characterization, and restoration. Workshop participants stated that once a deadly agent like *B. anthracis* has been identified, no further characterization is necessary until after building fumigation. During a decontamination event, a disinfectant must reach a specific dose (ppm-hours) to ensure efficacy. Post-fumigation sampling is performed to determine whether the dose has been delivered and the contaminating agent has presumably been eradicated. Some fumigators use biological indicators such as spores on steel coupons or paper strips, but the question remains how indicative are biological indicators of the condition of real world materials such as carpet, ceiling tiles, wood, painted walls, or fabric.

Standards for sampling decontaminated facilities are currently not available. CDC issued guidance (CDC 2002) on collecting environmental samples for culturing *B. anthracis* and established the Laboratory Response Network (LRN) to investigate and validate sampling and analytical methods for biological contaminants focusing on efficiency of surface sampling, air sampling, methods comparison, and variability. Research at the Edgewood Chemical Biological Center (ECBC) has been able to relate differences in kill efficacy to differences in surfaces, accounting for surface variability in real-world situations. The Department of Defense (DOD) is establishing an environmental LRN similar to that of the CDC to harmonize sampling.

Paula Krauter of Lawrence Livermore National Laboratory (LLNL) presented research on developing a 15-hour method for processing biological indicator strips using a real-

time polymerase chain reaction (PCR) technique called a rapid viability test protocol (RVTP) and compared it to a standard culture method which requires 7 days for results. The results were comparable except that the standard culture method reported a 1.5 % false positive rate while *the RVTP reported no false positives or negatives*.

Correct statistical design considers risk. The National Academy of Sciences (NAS 2005) addressed the question "How Clean is Safe?" in its publication "Reopening Public Facilities after a Biological Attack: A Decision–Making Framework." The NAS states that risk analysis informs interested parties of the probability of having any residual organisms in the building and of those residual organisms causing an infection in a human occupant, based on the detection limit, sampling efficiency, and dose-response data. They recommend convening an expert group and an Operations Working Group (OWG) composed of stakeholders, building managers, and decision makers to determine acceptable risk and whether a building can be declared safe for occupancy. If the risks cannot be determined with confidence because of high uncertainties associated with sampling or decontamination methods, the acceptable choice is to further decontaminate to increase the probability that the building is safe.

Decontamination of Biologically Contaminated Sites after a Flood Event

The issues of sampling and analysis of fungal microorganisms after a flood event are different from those after a terrorist attack. Generally a visual inspection leaves no doubt of the presence of mold, thus usually no sampling is indicated until after remediation work is completed. At that time the question then becomes what sampling and analysis plan will assure that the site is sufficiently remediated to occupy. Federal guidance provided by EPA's "Mold Remediation in Schools and Commercial Buildings" (USEPA 2001) and CDC's "Mold Prevention Strategies and Possible Health Effects in the Aftermath of Hurricanes Katrina and Rita" (CDC 2005) mainly defaults to industry experts.

The Institute of Inspection, Cleaning & Restoration Certification guide (IICRC 2003) defines indoor environmental conditions. A Condition 1 (normal fungal ecology) site may have settled spores, fungal fragments, or traces of actual growth whose identities, locations, and quantities are reflective of a normal fungal ecology for a similar indoor environment. In Condition 2 (settled spores) the site is primarily contaminated with settled spores that were dispersed directly or indirectly from a Condition 3 area, and may have traces of actual growth. A Condition 3 (actual growth) site is contaminated with the presence of actual mold growth and associated spores that may be active or dormant, visible or hidden. The IICRC recommends physical removal as the primary means of remediation to return the indoor environment to Condition 1 status and maintains that attempts to simply kill or encapsulate mold are not generally adequate. Physical removal also has the advantage that it mitigates fungal toxins (mycotoxins) and/or spore wall components (glucans) which are the most likely etiology of building related allergy/immunological complaints.

The IICRC guidance relies on Indoor Environmental Professionals (IEPs) as third party inspectors to assess and declare a building returned to Condition 1. The IEPs may consult

the American Conference of Governmental Industrial Hygienists guide (Macher 2005) for such assessment. Testing after mold remediation focuses on checking for removal of the water source and preparing an adequate sampling and analysis plan to capture the fungal ecology of the remediated space and comparing it to the "normal" fungal ecology for a similar indoor environment or to that immediately outdoors. If the remediated levels are higher or if the mold concentrations and species are significantly different, further remediation is required. In the absence of federal standards or recommendations for acceptable indoor airborne levels of viable mold, Baxter et al 2005 have attempted to devise criteria for differentiating "clean" from "moldy." Less than 1200 spores/m^3, <750 *Aspergillus/Penicillium* /m^3, or <1200 *Ascospores/Basidiospores* /m^3 constitutes clean by their definition.

EPA is researching ways to remediate mold-contaminated buildings without gutting them by testing the ability of chlorine dioxide gas to render fungi, their fragments, and their mycotoxins harmless. Such work generally requires DNA analysis using PCR. Work at Texas Tech (Wilson et al 2005) has shown that some fungi can be inactivated, but others remain toxic.

Testing and Reporting the Efficiency of In-Duct UV Light Technologies

Testing a UV light device installed in a test rig that represents the ductwork of an HVAC system is generally straightforward. The device is challenged with bioaerosols and the air is sampled upstream and downstream of the device getting 6 to 12 samples for each measurement. Each sample is plated and grown out and the CFUs are counted. The counts for all the measurements are summed and the penetration and efficiency calculations are performed using the sum. A problem occurs when the sum of the counts is zero, as occurs when the microorganisms are very efficiently killed. Non-detects do not necessarily mean zero microorganisms, therefore 100% efficiency for the device is not reported when the outcome of downstream tests is *complete non-detection* of organisms. How then should the calculation be handled?

Restructuring the test to get a few counts downstream should be considered, however providing more bioaerosol concentration upstream generally will not affect downstream counts and neither will longer downstream sampling times or plating more sample. The solution is to consider that these low downstream numbers have a Poisson distribution and to use a Poisson statistics table to provide a number that is an upper bound for any sum of counts less than 50 (including zero). Poisson statistics have the unique property that the sum of Poisson averages is still Poisson. Thus an efficiency can be reported that is "greater than" the calculated value, generally >99%.

Conclusions

The traditional thought processes that govern planning for sampling, analysis, and detection limits of microorganisms are somewhat different from those for non-biological substances. To analyze risk the sampling plan should consider probability as well as targeted sampler siting. Both viable and non-viable microbes and their products should be collected and analyzed. Research on molecular methods is improving the science of

identification of microorganisms. The human element is often as important as the science. For people to be comfortable living or working in a building after a contamination and subsequent decontamination event, stakeholders must be drawn in and educated at the outset and throughout the process.

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