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technical BRIEF

BUILDING A SCIENTIFIC FOUNDATION FOR SOUND ENVIRONMENTAL DECISIONS

Persistence of Categories A and B Select Agents in Environmental Matrices

INTRODUCTION

Persistence is the ability of an organism to remain viable over time under a given environmental condition. For bacteria, viability is the ability to remain alive, whereas for viruses, it is the ability to remain infectious. Understanding a biological agent's ability to persist in the environment would help authorities to properly assess and respond in the event of the agent's release.

The purpose of this brief is to summarize persistence data documented in an EPA literature review (U.S. EPA, 2014a) entitled Persistence of Categories A and B Select Agents in Environmental Matrices (EPA/600/R-14/074). Category A biological agents cause high rates of mortality and are easily disseminated or easily transmitted from person to person. Category B agents cause illness with low mortality and are moderately easy to disseminate. Assessment of persistence data is important to (1) allow informed emergency response and remediation decisions following a contamination incident, and (2) identify gaps in the current state of the science so research can be focused toward closing these gaps.

This brief provides a short summary of the open literature findings published to date, specifically for biological agent persistence. These data cover numerous agents, as well as various environmental conditions and surface types. This brief reports the longest duration of persistence, which is from the time of inoculation to the last sampling interval that produced viable organisms. Several studies, however, did not continue until organism could no longer be cultured; in those cases, the pathogen's actual duration of persistence could not be determined.

"*Biological agent* means any microorganism (including, but not limited to, bacteria, viruses, fungi, rickettsiae, or protozoa), or infectious substance, or any naturally occurring, bioengineered, or synthesized component of any such microorganism or infectious substance, capable of causing death, disease, or other biological malfunction in a human, an animal, a plant, or another living organism; deterioration of food, water, equipment, supplies, or material of any kind; or deleterious alteration of the environment."

42 Code of Federal Regulations Part 73

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CONDITIONS AFFECTING PERSISTENCE

The literature review documents the duration of persistence of biological agents and provides information on environmental conditions and species interactions that either favor or hinder persistence. The environmental media considered were aerosol, soil, water, or fomite (an object, surface, or substance that can transmit pathogens). The review also identified the gaps in the scientific literature that impede a thorough understanding of the persistence of these pathogens. This summary, based on the literature review, describes what is known and what is unknown about these conditions and interactions.

As noted below, some species enter into a state where they are metabolically active, yet not culturable, with unknown viability. Ducret et al., 2014, concluded that the physiological significance of the state is unknown and controversial; it could be an adaptive state favoring long-term survival, it could be a deteriorating state leading to death, or it could be an injured state that prevents organisms from being cultured. The ability of an agent to subsequently cause infection or induce human disease following release into the environment was beyond the scope of the review. The importance of this metabolic state is an area in need of additional research.

Bacillus anthracis

B. anthracis is a spore-forming bacterial Category-A select agent, the causative agent of the disease anthrax. Under appropriate conditions, *B. anthracis* can form protective biofilms on glass and polyvinyl surfaces covered with nutrient media. *B. anthracis* strains involved in gut colonization have also formed biofilms. However, conditions and the overall persistence of the formed biofilms have yet to be explored.

B. anthracis spores can persist in soil for many years. Many symbiotic relationships between *Bacillus* species and soil-borne organisms have been reported in the literature. *B. anthracis* has been found to geminate in amoeba, and to colonize the hindgut of earthworms. There is some evidence that the interaction between earthworms and *B. anthracis* might be dependent on the presence of bacteriophages. It is unknown whether the colonies formed in the hindgut of earthworms and the spores that germinate in amoeba can result in increased numbers of spores in the environment or if these spores would be virulent.

The *B. anthracis* lifecycle and the earthworm lifecycle in the soil have similarities. Both prefer alkaline, calcium rich, organic soils. In addition, anthrax outbreaks often occur following flooding, which is also when earthworms tend to migrate to the surface. The post-flooding anthrax outbreaks could be due to *B. anthracis* spores being carried upward to the soil surface and onto vegetation via colonized earthworm digestive systems. (For more information on the persistence of *B. anthracis* spores in soil, see U.S. EPA, 2014b, entitled *Literature Review on Mechanisms that Affect Persistence of Bacillus anthracis in Soils*.)

The literature search included persistence on surfaces, but little data on persistence on surfaces was found. Testing on fomites was not conducted at environmental conditions <25°C, <80% RH, or with simulated sunlight.

Virulent *B. anthracis* contains two plasmids, pX01 and pX02. The pX01 plasmid has been found to be required for spore germination within the amoeba. For strains lacking pX01, bacteriophage infection of *B. anthracis* could restore functional gene activity necessary to survive and replicate in earthworms, rhizosphere, biofilms, and soil. Researchers have hypothesized that pX02 is lacking in multiple natural *B. anthracis* strains because it is not required for proliferation. However, there is little information in the literature on conditions that promote or inhibit proliferation.

Some studies have found that spores can germinate and propagate in the rhizosphere of a common pasture grass, but do not germinate without the grass. Conflicting studies suggest that presence of grass does not increase survival or multiplication of *B. anthracis* in soil, but serves to promote transmission of *B. anthracis* to grazing hosts by attracting more hosts to the area. Further research is needed to determine if *B. anthracis* can take up the pX02 plasmid in the environment and thus regain virulence.

Brucella species

Brucella species are non-spore-forming bacterial Category-B select agents, which cause the disease brucellosis*. Brucella* species can enter a state where they are metabolically active, yet not culturable, with unknown viability. *Brucella melitensis* is typically associated with goats, *Brucella suis* is associated with swine, and *Brucella abortus* is often associated with cattle. All three species can infect humans.

The persistence of *B. melitensis* and *B. suis* in water (<9 days at 5°C and 25°C) was considerably lower than the persistence of *B. abortus* in water (>57 days at 8°C and 20°C). It is uncertain if the difference is attributed to species-level differences or the study parameters.

Under certain conditions (e.g., nutritionally deficient, low oxygen) *B. abortus* was found to aggregate and form biofilms, which can enhance the bacteria's tolerance to desiccation. *B. abortus* appeared to survive better in moist soil than dry soil (66 days in wet soil compared to <4 days in dried soil).

Brucella species can survive for several weeks in water, but the bacteria are sensitive to heat, with < I day survival at 37° C in lake water. *Brucella* species also appear to be adversely affected by exposure to sunlight. No data on the survival of *Brucella* species in aerosols was found during this review. Research is needed on the mechanisms that contribute to the environmental persistence of *Brucella* species including species-specific data.

Burkholderia mallei

B. mallei is a non-spore-forming non-motile bacterial category-B select agent, which causes the disease glanders. *B. mallei* can enter a state where they are metabolically active, yet not culturable, with unknown viability. *B. mallei* is primarily associated with horses and is not expected to survive outside a host for long durations. However, *B. mallei* "has an affinity for warm and moist conditions and may survive for up to 3 months in stable bedding, manure, feed and water troughs (particularly if heated), wastewater and equine transporters. Although three persistence studies were identified with *B. mallei* in water, the results were somewhat conflicting.

No data on the survival of *B. mallei* in aerosols or on soil was found during this review. Information *on B. mallei* persistence on fomites found during this review was not supported with specific data or laboratory controlled studies.

Burkholderia pseudomallei

B. pseudomallei is a non-spore-forming motile bacterial category-B select agent, which causes the disease melioidosis. *B. pseudomallei* can enter a state where they are metabolically active, yet not culturable, with unknown viability. *B. pseudomallei* is associated with water and with soil. *B. pseudomallei* occurs in tropical and sub-tropical climates and is associated with decaying organic matter in the environment. *B. pseudomallei* can grow in anoxic (oxygen deficient) environments.

Interactions with other organisms can influence persistence. *B. pseudomallei* is associated with the rhizosphere, roots, and above ground parts of various grasses, especially non-native grasses introduced for grazing animals. The relationship between *B. pseudomallei* and vegetation is not well understood. Other soil bacteria (e.g., *Burkholderia multivorans*) could inhibit *B. pseudomallei* growth. On the other hand, *B. pseudomallei* can form biofilms and survive in amoeba cysts and fungi.

Survival in various media is not well understood. *B. pseudomallei* can be transported by aerosols and has been isolated from aerator spray associated with a water treatment plant. However, no data on the survival durations of *B. pseudomallei* in aerosols was found during this review. Only one study investigated the persistence of *B. pseudomallei* on fomites. However, the influence of different environmental conditions (temperature and humidity) on survival on fomites was not investigated. Environmental factors dictating the occurrence of *B. pseudomallei* in soil and water are not well established.

Similarly, survival in soil and the effect of sunlight is not well understood. Subsurface samples (e.g., at a 25-60 centimeter [cm] depth) are more likely to yield *B. pseudomallei* than surface soil. *B. pseudomallei* is infrequently found in surface soil, possibly because the organism might be adversely affected by sunlight. However, the review found no definitive studies on the effects of sunlight on *B. pseudomallei* persistence. It is known that *B. pseudomallei* were killed

by radiation from an UV lamp (465 microwatt [µW]/square centimeter [cm2] for 7.75 minutes), but radiation associated with natural sunlight can be absorbed by other materials thereby limiting sunlight's killing effect.

Other factors also affect persistence in soils. Optimal temperatures for *B. pseudomallei* growth in soil are 37°C to 42°C, although some strains were able to grow at 4°C. Optimal soil pH was 6.5 to 7.5. *B. pseudomallei* appears to benefit from moist soil, but can persist in soil that gradually dries. *B. pseudomallei* was not recovered following inoculation into dry soil, but was recovered from dry soil in farmed areas suggesting that factors other than moisture are needed to support *B. pseudomallei* growth. Under conditions of low water content, organic matter in the soil might enable *B. pseudomallei* to survive.

Coxiella burnetii

C. burnetii is an obligate intracellular bacterial category-B select agent, which causes Q fever. Commonly, barnyard dust from infected cattle, sheep, and goats transmits the disease to humans. *C. burnetii* can be aerosolized and transported by wind long distances. *C. burnetii* is not known to be strongly affected by high or low temperatures, drought, or humidity levels. No data on the persistence of *C. burnetii* in water was found during this review.

There are a number of environmental reservoirs that could aid in the persistence and transmission of *C. burnetii. C. burnetii* can invade a variety of hosts including amoebae, ticks, birds, and mammals. It appears likely that these hosts facilitate the dissemination of the bacterium throughout the environment. *C. burnetii* was able to survive within amoeba for 6 weeks; soil amoeba could provide an intracellular niche for the survival of *C. burnetii* in a spore-like form. Epidemiological evidence suggests that *C. burnetii* may be carried over considerable distances on fomites.

There are uncertainties and unknowns in the information on persistence for *C. burnetii*. The bacterium may exist as "large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC)" physiological forms, with SCV and SDC being the environmentally persistent forms. Persistence might vary by the form of *C. burnetii* excreted from the host, but the specific forms excreted in milk, feces or placentas are unknown. In addition, much of the persistence data of *C. burnetii* (in aerosol and fomites studies, and in some soil studies) were based on environmental sampling associated with Q fever outbreaks and viability was not assessed. Also, the studies were not conducted in controlled laboratory settings, so recontamination/cross-contamination could have occurred.

Francisella tularensis

F. tularensis is a non-spore-forming bacterial category-A select agent, which causes tularemia, also called rabbit fever. *F. tularensis* can enter into a state where they are metabolically active, yet not culturable, with unknown viability. *F. tularensis* can infect mammals, arthropods, and

protozoans. Virulent strains of *F. tularensis* were shown to survive in the cysts of *Acanthamoeba castellanii* for at least 3 weeks post-infection.

F. tularensis can survive in diverse environments, but the mechanism by which *F. tularensis* persists or establishes an environmental reservoir following a release is unknown. *F. tularensis* can survive and reproduce in water for relatively long periods of time (including waters with high nutrient levels and protozoan predation), although virulence may be lost. *F. tularensis* survived longer in brackish water than fresh water. The salt and sulfur content of the brackish water may have contributed survival; sulfur-containing amino acids, cysteine or cystine, are usually required for the cultivation of *F. tularensis*. Sodium chloride also enhances *F. tularensis* culture growth. By forming a biofilm in natural ecosystems, *Francisella* might be able to survive the environmental conditions of mud and waterways, and forming a biofilm could be a mechanism for persistence within the ticks.

F. tularensis subspecies *holarctica*, which is found more widely in the northern hemisphere, is associated with water-borne disease and is transmitted by mosquitoes, ticks, and biting flies. No information is available on its persistence in soil.

Unidentified constituents in the air (possibly olefins from oil refineries and dense car populations) may reduce *F. tularensis* viability. No information in the influence of solar radiation (UV light) on *F. tularensis* persistence.

Viral Encephalitis and Hemorrhagic Fever Agents

Encephalitis viruses (category B) and hemorrhagic fever viruses (category A) are viral agents. Viruses for which persistence was studied include viral hemorrhagic fever filoviruses (e.g., Ebola and Marburg) and arenaviruses (e.g., Lassa and Machupo), flaviviruses (Japanese encephalitis virus, St. Louis encephalitis virus, and yellow fever virus), bunyaviruses (hantavirus and Crimean-Congo virus), and alphaviruses (Venezuelan equine encephalitis [VEE]).

Japanese encephalitis virus and VEE virus appeared to have inverse relationships with relative humidity (RH) in aerosols. Persistence data were only identified for viral hemorrhagic fever agents in aerosols and on fomites.

Persistence data on viral encephalitis and hemorrhagic fever agents is sparse. No data were identified on the persistence of viral encephalitis or hemorrhagic fever agents in soil. Persistence studies on viral encephalitis and hemorrhagic fever agents in aerosols or on fomites were not continued until attempts to culture produced no viable organism. So, the longest duration that these agents could survive as aerosols or on surfaces remains unknown. Studies in water were limited to VEE virus. None of the studies identified for viral encephalitis and hemorrhagic fever agents assessed the impact of sunlight on persistence.

Yersinia pestis

Y. pestis is a non-spore-forming non-motile bacterial category-A agent, which causes plague. *Y. pestis* can enter a state where they are metabolically active, yet not culturable, with unknown viability in water. *Y. pestis* is also possibly survives inside amoeba. Most persistence data on *Y. pestis* were associated with survivability in water. *Y. pestis* persistence in water ranged from days to years with no apparent relationships between environmental conditions and persistence. Variability associated with *Y. pestis* persistence in water might be attributed to differences in water chemistry, organism strain or growth phase, growth media for culturing and recovery, and inoculum levels.

Few studies were found focusing on the impact of various environmental parameters (e.g., humidity, sunlight, and temperature) on persistence. Little data were identified on the persistence of *Y. pestis* in aerosols and fomites.

RANGE OF PERSISTENCE

The range of persistence durations are summarized by agent and medium in Table 1. The table also includes the environmental condition associated with each value and identifies agent/medium combinations where persistence data are lacking. Persistence was found to be highly variable between species and subspecies. Persistence was also found to be affected by various environmental media and factors (e.g., temperature, relative humidity (RH), and sunlight), preparation and application methods, and nutrient conditions. Persistence can increase in the presence of organisms that serve as hosts or symbionts (e.g., amoeba, earthworms); persistence can decrease in the presence of competing and/or predatory organisms. Differing analytical methods also affect comparison or interpretation of persistence results across studies (e.g., different culture media, incubation temperatures and times). Similarly, the estimates of persistence may differ depending on the technique used (molecular, culturing, counting, etc.).

Table 1. Agent Persistence in the Environment

Agent	Medium	Shortest Duration Reported			Longest Duration Reported		
		Persistence Duration	Associated Environmental Condition	Reference*	Persistence Duration	Associated Environmental Condition	Reference
*Bacillus anthracis**	Aerosol	--			--		
	Fomite	6 hours	25°C, 80% RH on stainless steel coated with silver and zinc zeolite paint	Galeano et al. (2003)	7 days†	37°C on polystyrene and glass as a biofilm in BHI broth	Lee et al. (2007)
	Soil	96 hours(veg)	Topsoil RH 46%, UV and no UV	U.S. EPA, 2014	40 years (spore)		Manchee et al. [1994)
					120 hours (veg)	Topsoil, RH 46%,no UV	U.S. EPA, 2014c
					100 hours(veg)	Topsoil, RH 60% (approx.) simulated sunlight	U.S. EPA, 2014c
	Water	3 days	Distilled water	Sinclair et al. (2008)	6 days	Water	Sinclair et al. (2008)
Brucella species	Aerosol	--			--		
	Fomite	4 hours	22°C, 45% RH on painted joint tape	Ryan (2010)	56 days†	22°C, 40% RH on aluminum and glass; and 5°C, 30% RH on aluminum, glass, and wood	Calfee and Wendling (2012)
	Soil	<4 days	Dried soil	Nicoletti (1980)	43 days	Bison partition sites in Greater Yellowstone, identified in April	Aune et al. (2012)
	Water	<1 day	37°C	Nicoletti (1980)	77 days	Room temperature	Nicoletti (1980)
Burkholderia mallei	Aerosol	--			--		
	Fomite	3 weeks	Environmental survival (specific fomites not identified) in wet, humid, or dark conditions	Dvorak and Spickler (2008)	3 months	On stable bedding, troughs, and harness equipment	Malik et al. (2012)
	Soil	--			--		
	Water	1 day	Dechlorinated municipal water, 5°C and 25°C	Gilbert and Rose (2012)	28 days	Tap water, room temperature	Miller et al. (1948)

Burkholderia pseudomallei	Aerosol	--			--		
	Fomite	6 hours	Applied in Butterfield buffer to glass, paper, polyethylene, and stainless steel; and applied in BHI broth to stainless steel	Shams et al. (2007)	3 days	Applied in BHI broth to paper, polyethylene, and stainless steel	Shams et al. (2007)
	Soil	<10 days	Soil inoculated with antagonistic bacteria (e.g., *B. multivorans*)	Lin et al. (2011)	30 months	Soil stored in plastic bags at ambient temperature (13°C to 33°C).	Thomas and Forbes-Faulkner (1981)
	Water	60 minutes	Water exposed to sunlight	Sagripanti et al. (2009)	16 years†	Distilled water, 25°C	Pumpuang et al. (2011)
Coxiella burnetii	Aerosol	--			--		
	Fomite	--			--		
	Soil	20 days†	20°C, 4°C, and -20°C	Evstigneeva et al. (2007)	20 days†	20°C, 4°C, and -20°C	Evstigneeva et al. (2007)
	Water	--			--		
Francisella tularensis	Aerosol	29 minutes (T_{99} value) §	50% RH, wet dissemination	Sinclair et al. (2008); Cox (1971); Cox and Goldberg (1972)	5 days	90% RH, wet dissemination	Sinclair et al. (2008); Cox (1971); Cox and Goldberg (1972)
	Fomite	20 minutes	Desiccated on filter paper	Faith et al. (2012)	16 days†	37°C, 0% RH on stainless steel	Wilkinson (1966)
	Soil	--			--		
	Water	<1 day	Dechlorinated municipal water, 5°C and 25°C	Gilbert and Rose (2012)	34 days†	Brackish-water, 21°C	Berrada and Telford (2011)

Agent	Medium	Shortest Duration Reported			Longest Duration Reported		
		Persistence Duration	Associated Environmental Condition	Reference	Persistence Duration	Associated Environmental Condition	Reference
Viral encephalitis and hemorrhagic fever agents	Aerosol	1 hour†	22°C, 50% RH	Smither et al. (2011)	120 days (T_{99} value)	21°C, 23% RH	Sinclair et al. (2008)
	Fomite	5 minutes	Desiccated, 37°C	Fogarty et al. (2008)	50 days†	4°C, 55% RH in tissue culture medium on glass	Piercy et al. (2010)
	Soil					--	
	Water	<60 minutes	Tap water (1 mg L^{-1} free available chlorine or 2 mg L^{-1} total bromine), 21°C	Wade et al. (2010)	69 days (T_{99} value)	4°C	Sinclair et al. (2008)
Yersinia pestis	Aerosol	34 minutes (T_{99} value)	26°C, 87% RH	Sinclair et al. (2008)	57 minutes (T_{99} value)	26°C, 50% RH	Sinclair et al. (2008)
	Fomite	7 minutes (T_{99} value)	30°C, 52% RH on metal (stainless steel)	Sinclair et al. (2008); Wilkinson (1966)	7 days†	20°C, 50% RH on aluminum and painted joint tape	Ryan (2010)
	Soil	24 days†	Arizona soil during late October in an area with limited exposure to UV light	Eisen et al. (2008)	10 months†	4°C to 8°C	Sinclair et al. (2008)
	Water	1 day	Dechlorinated municipal water, 5°C	Gilbert and Rose (2012)	3 years†	Autoclaved river water	Pawlowski et al. (2011)

BHI, brain-heart infusion; RH, relative humidity; uv, ultraviolet; veg, vegetative state.

-- Not tested/not reported.

* This review focused on vegetative *B. anthracis* only.

† The longest duration tested (i.e., the actual persistence duration could be longer).

§ The time required for the microbial count to decrease by 99%

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