POST GRADUATE COURSES
(Partial)

S-Plus Data Analysis, MathSoft, 6/94.
Air Pollutant Emission Calculations, UC Berkeley Extension, 6-7/94
Assessment, Control and Remediation of LNAPL Contaminated Sites, API and USEPA, 9/94
Pesticides in the TIE Process, SETAC, 6/96
Sulfate Minerals: Geochemistry, Crystallography, and Environmental Significance, Mineralogical Society of America/Geochemical Society, 11/00.
Design of Gas Turbine Combined Cycle and Cogeneration Systems, Thermoflow, 12/00
Air-Cooled Steam Condensers and Dry- and Hybrid-Cooling Towers, Power-Gen, 12/01
Combustion Turbine Power Augmentation with Inlet Cooling and Wet Compression, Power-Gen, 12/01
CEQA Update, UC Berkeley Extension, 3/02
The Health Effects of Chemicals, Drugs, and Pollutants, UC Berkeley Extension, 4-5/02
Noise Exposure Assessment: Sampling Strategy and Data Acquisition, AIHA PDC 205, 6/02
Noise Exposure Measurement Instruments and Techniques, AIHA PDC 302, 6/02
Noise Control Engineering, AIHA PDC 432, 6/02
Optimizing Generation and Air Emissions, Power-Gen, 12/02
Utility Industry Issues, Power-Gen, 12/02
Multipollutant Emission Control, Coal-Gen, 8/03
Community Noise, AIHA PDC 104, 5/04
Cutting-Edge Topics in Noise and Hearing Conservation, AIHA 5/04
Selective Catalytic Reduction: From Planning to Operation, Power-Gen, 12/05
Improving the FGD Decision Process, Power-Gen, 12/05
E-Discovery, CEB, 6/06
McIlvaine Hot Topic Hour, FGD Project Delay Factors, 8/10/06
McIlvaine Hot Topic Hour, What Mercury Technologies Are Available, 9/14/06
McIlvaine Hot Topic Hour, SCR Catalyst Choices, 10/12/06
McIlvaine Hot Topic Hour, Particulate Choices for Low Sulfur Coal, 10/19/06
McIlvaine Hot Topic Hour, Impact of PM2.5 on Power Plant Choices, 11/2/06
McIlvaine Hot Topic Hour, Dry Scrubbers, 11/9/06
Cost Estimating and Tricks of the Trade – A Practical Approach, PDH P159, 11/19/06
Process Equipment Cost Estimating by Ratio & Proportion, PDH G127 11/19/06
Power Plant Air Quality Decisions, Power-Gen 11/06
McIlvaine Hot Topic Hour, WE Energies Hg Control Update, 1/12/07
Negotiating Permit Conditions, EEUC, 1/21/07
BACT for Utilities, EEUC, 1/21/07
McIlvaine Hot Topic Hour, Chinese FGD/SCR Program & Impact on World, 2/1/07
McIlvaine Hot Topic Hour, Mercury Control Cost & Performance, 2/15/07
McIlvaine Hot Topic Hour, Mercury CEMS, 4/12/07

Coal-to-Liquids – A Timely Revival, 9th Electric Power, 4/30/07
Advances in Multi-Pollutant and CO_2 Control Technologies, 9th Electric Power, 4/30/07
McIlvaine Hot Topic Hour, Measurement & Control of PM2.5, 5/17/07
McIlvaine Hot Topic Hour, Co-firing and Gasifying Biomass, 5/31/07
McIlvaine Hot Topic Hour, Mercury Cost and Performance, 6/14/07
Ethanol 101: Points to Consider When Building an Ethanol Plant, BBI International, 6/26/07
Low Cost Optimization of Flue Gas Desulfurization Equipment, Fluent, Inc., 7/6/07.
McIlvaine Hot Topic Hour, CEMS for Measurement of NH3, SO3, Low NOx, 7/12/07
McIlvaine Hot Topic Hour, Mercury Removal Status & Cost, 8/9/07
McIlvaine Hot Topic Hour, Filter Media Selection for Coal-Fired Boilers, 9/13/07
McIlvaine Hot Topic Hour, Catalyst Performance on NOx, SO3, Mercury, 10/11/07
PRB Coal Users Group, PRB 101, 12/4/07
McIlvaine Hot Topic Hour, Mercury Control Update, 10/25/07
Circulating Fluidized Bed Boilers, Their Operation, Control and Optimization, Power-Gen, 12/8/07
Renewable Energy Credits & Greenhouse Gas Offsets, Power-Gen, 12/9/07
Petroleum Engineering & Petroleum Downstream Marketing, PDH K117, 1/5/08
Estimating Greenhouse Gas Emissions from Manufacturing, PDH C191, 1/6/08
McIlvaine Hot Topic Hour, NOx Reagents, 1/17/08
McIlvaine Hot Topic Hour, Mercury Control, 1/31/08
McIlvaine Hot Topic Hour, Mercury Monitoring, 3/6/08
McIlvaine Hot Topic Hour, SCR Catalysts, 3/13/08
Argus 2008 Climate Policy Outlook, 3/26/08
Argus Pet Coke Supply and Demand 2008, 3/27/08
McIlvaine Hot Topic Hour, SO3 Issues and Answers, 3/27/08
McIlvaine Hot Topic Hour, Mercury Control, 4/24/08
McIlvaine Hot Topic Hour, Co-Firing Biomass, 5/1/08
McIlvaine Hot Topic Hour, Coal Gasification, 6/5/08
McIlvaine Hot Topic Hour, Spray Driers vs. CFBs, 7/3/08
McIlvaine Hot Topic Hour, Air Pollution Control Cost Escalation, 9/25/08
McIlvaine Hot Topic Hour, Greenhouse Gas Strategies for Coal Fired Power Plant Operators, 10/2/08
McIlvaine Hot Topic Hour, Mercury and Toxics Monitoring, 2/5/09
McIlvaine Hot Topic Hour, Dry Precipitator Efficiency Improvements, 2/12/09
McIlvaine Hot Topic Hour, Coal Selection & Impact on Emissions, 2/26/09
McIlvaine Hot Topic Hour, 98% Limestone Scrubber Efficiency, 7/9/09
McIlvaine Hot Topic Hour, Carbon Management Strategies and Technologies, 6/24/10
McIlvaine Hot Topic Hour, Gas Turbine O&M, 7/22/10

McIlvaine Hot Topic Hour, Industrial Boiler MACT – Impact and Control Options, March 10, 2011
McIlvaine Hot Topic Hour, Fuel Impacts on SCR Catalysts, June 30, 2011.

Interest Rates, PDH P204, 3/9/12
Mechanics Liens, PDHOnline, 2/24/13.
Understanding Concerns with Dry Sorbent Injection as a Coal Plant Pollution Control, Webinar #874-567-839 by Cleanenergy.Org, March 4, 2013
Webinar: Coal-to-Gas Switching: What You Need to Know to Make the Investment, sponsored by PennWell Power Engineering Magazine, March 14, 2013. Available at: https://event.webcasts.com/viewer/event.jsp?ei=1013472.

Feature

Unsustainable Wind Turbine Blade Disposal Practices in the United States: A Case for Policy Intervention and Technological Innovation

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Abstract

Finding ways to manage the waste from the expected high number of wind turbine blades in need of disposal is crucial to harvest wind energy in a truly sustainable manner. Landfilling is the most cost-effective disposal method in the United States, but it imposes significant environmental impacts. Thermal, mechanical, and chemical processes allow for some energy and/or material recovery, but they also carry potential negative externalities. This article explores the main economic and environmental issues with various wind turbine blade disposal methods. We argue for the necessity of policy intervention that encourages industry to develop better technologies to make wind turbine blade disposal sustainable, both environmentally and economically. We present some of the technological initiatives being researched, such as the use of bio-derived resins and thermoplastic composites in the manufacturing process of the blades.

Keywords

wind energy, recycling, sustainability, environment, occupational safety

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Introduction

Globally, more than seventy thousand wind turbine blades were deployed in 2012[1] and there were 433 gigawatts (GW) of wind installed capacity worldwide at the end of 2015.[2] Moreover, the United States' installed wind power capacity will need to increase from 74 GW to 300 GW[3] to achieve its 20% wind production goal by 2030.[3] To meet the increasing demand, not only are more blades being manufactured, but also blades of up to 100 meters long are being designed and produced.[4] The wind turbine blades are designed to have a lifespan of about twenty years, after which they would have to be dismantled due to physical degradation or damage beyond repair. Furthermore, constant development of more efficient blades with higher power generation capacity is resulting in blade replacement well before the twenty-year life span.[5] Estimations have suggested that between 330,000 tons/year by 2028 and 418,000 tons/year by 2040 of composite material from blades will need to be disposed worldwide.[6] That would be equivalent to the amount of plastics waste generated by four million people in the United States in 2013.[7] This anticipated increase in blade manufacturing and disposal will likely lead to adverse environmental consequences, as well as potential occupational exposures, especially because available technologies and key economic constraints result in undesirable disposal methods as the only feasible options.

The material in the shells of the wind turbine blades is typically glass fiber-reinforced polymer (GFRP), a resin-matrix material reinforced with fiberglass. In particular, the shells are commonly made from a combination of epoxy resin and glass fiber reinforcement.[8] The blades also contain sandwiched core materials such as polyvinyl chloride foam, polyethylene terephthalate foam, or balsa wood, as well as bonded joints, coatings (polyurethane), and lightning conductors.[8] Conventional epoxy resins are thermosetting materials usually produced by a reaction of epichlorohydrin and bisphenol A in the presence of sodium hydroxide.[9] Both bisphenol A and epichlorohydrin are derived from petrochemicals. Contrary to other types, once cured, thermoset polymers cannot be melted and reshaped by applying heat at high temperatures. As a result, thermoset composites cannot be reformed by any means other than machining, which risks compromising the properties of the material through damage or destruction of the reinforcing fibers. Therefore, the GFRP found in the blades poses a challenge to find or develop more sustainable end-of-life alternatives.

The issue of wind turbine blade disposal had received little attention until recently when some of the oldest wind farms in the United States were in need of ways to dispose of blades that have reached the end of their usefulness. Currently, there are more than 100 wind farms in the United States that are fifteen years or older, with more than 2.38 GW of installed capacity.[10] Since the useful life of a blade is about fifteen to twenty years, these wind farms will likely need to replace a large number of blades in the near future. The United States' installed wind power capacity reached 74 GW at the end of 2015.[11] By using the

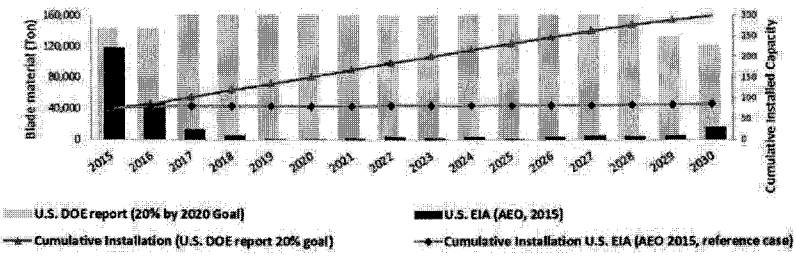

Figure 1. Estimated amount of blade material to be used per year (tons) and cumulative installed capacity (GW).
Source: U.S. Department of Energy,[3] American Wind Energy Association,[11] and U.S. Energy Information Administration.[13]

National Renewable Energy Laboratory 5-Megawatt reference wind turbine,[12] we estimate that about 10 kg of material is used per kilowatt of capacity, which means we will have about 728,000 tons of blade material to dispose of over the next twenty years. Disposing of this amount of material from existing capacity in a sustainable way will be challenging given the current technological and economic limits of available methods.

That amount of material does not include future wind power capacity installation, nor does it take into account blade replacement before the twenty-year life span. Both situations will serve to further increase the disposal challenge. Figure 1 shows projections of the estimated wind turbine blade material to be used in the United States wind industry annually over the next fifteen years. The same assumption of 10 kg of blade material per each kilowatt of installed capacity was used. Based on projections of wind installed capacity from the Annual Energy Outlook 2015,[13] we estimate that an average of approximately 15,000 tons of blade material will be used between 2015 and 2030 annually. The projection used is based on the reference case, which assumes that current laws and regulations remain unchanged and that about 35 GW of wind capacity would be added between 2015 and 2040. However, the U.S. Department of Energy's (DOE) projection suggests that the country will need to increase its wind energy installed capacity by an average of approximately 14 GW annually if it wants to meet the goal of 20% wind energy by 2030.[3] The projection was made in 2008 and no revision has been made since then, but a 2015 report by the U.S. DOE[14] suggests that with proper policy, the scenario of 20% by 2030 is feasible. In that case, we estimate that an average of about 165,000 tons of blade material would be produced annually.

Overview of Concerns

The excellent stability of the GFRP material found in wind turbine blades has challenged the development of an optimal waste management method.

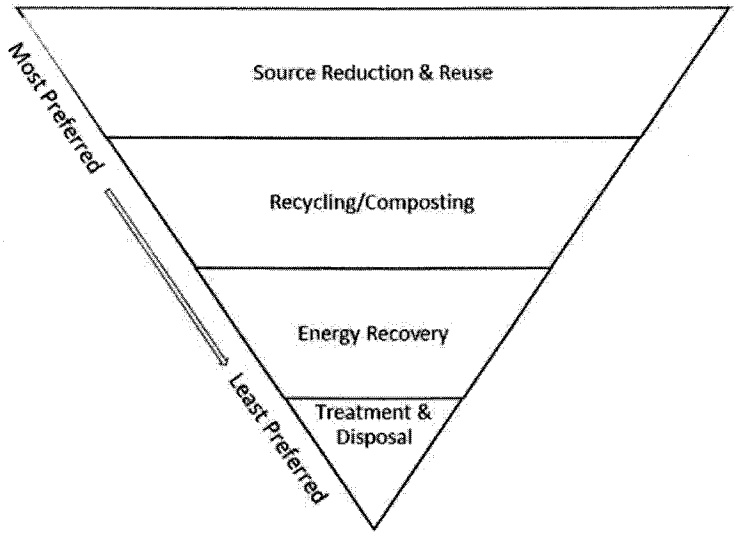

Figure 2. Non-hazardous waste management hierarchy.
Source: U.S. Environmental Protection Agency.[15]

According to the U.S. DOE's nonhazardous waste management hierarchy, source reduction and reuse, which refers to waste prevention and reutilization of items, is preferred to recycling (see Figure 2).[15] That is because recycling is a more energy-intensive process that entails collecting, sorting, processing, and remanufacturing what would otherwise be considered waste material.[15] Currently, there are different disposal methods for the GFRP found in the wind turbine blades, some of which are considered recycling methods whenever some recovery of energy and/or material is possible. Each method, however, carries negative environmental and economic implications, as well as potential occupational hazards.

Table 1 provides a summary of the existing disposal methods and their key concerns. Despite its negative consequences, landfilling has so far been the most commonly utilized wind turbine blade disposal method. Given that cured epoxy resin is present in the blades, landfilling is especially problematic because its high resistance to heat, sunlight, and moisture[16] means that it will take hundreds of years to degrade in a landfill environment. The wood and other organic material present in the blades would also end up in landfills, potentially releasing methane, a potent greenhouse gas, and other volatile organic compounds to the environment.[17] Even though gas collection systems that capture the methane released in landfills and use it as an energy source are common, a significant percent of the methane generated inevitably escapes depending on the overall efficiency of the system.[17] While methane emission from the balsa wood used in

Table 1. Existing disposal methods and some of their main concerns.

Disposal method	Economic	Environment and occupational exposure
Landfill	Opportunity cost of unrecovered material and concerns of long-term space availability	Release of methane and other volatile organic compounds from wood and other organics in the blades
Incineration with energy and/or material recovery	Significant energy and machinery requirements to cut and transport the blades to the incineration plant	Pollutant ash after the incineration process, possible emissions of hazardous flue gasses, and potential hazards from mechanical processing
Pyrolysis	Low economic viability because of degradation of resulting fibers	Emission of environmentally hazardous off-gasses and potential hazards from mechanical processing
Fluidized bed combustion	Low economic viability because of degradation of resulting fibers	Potential hazards from mechanical processing
Chemical	Economic viability dependent on chemical process used	Use of hazardous chemicals and dust from mechanical processing of the blades
Mechanical	Low market value of both the resulting fibers and substitute virgin material	Dust emission during the grinding process of glass fiber thermoset composites

Note. Courtesy of authors. See text for sources.

one blade would be insignificant compared with the alternative of burning fossil fuel to generate the same amount of electricity that a blade would generate,[a] other alternatives (e.g., composting) would be preferred from a long-term sustainability perspective. Likewise, landfilling creates issues of long-term space availability. Although landfill capacity appears adequate nationally, it is limited in some areas[19] and will likely be problematic in space-constrained states. It also carries an opportunity cost of unrecovered energy and material from the blades, some of which can be potentially recovered with other disposal technologies presented in Table 1.

Landfilling wind turbine blades at the end of their useful life is particularly appealing in the United States due to the amount of land available for disposal

sites, which makes it the cheapest option. Unfortunately, the cost efficiency of landfill use also makes it very difficult for alternative disposal methods to compete. Conversely, many European countries have banned or reduced the landfilling of FRPs because of the high content of organic material, and as a result, alternative disposal methods are being explored.[20] Based on information obtained from phone interviews with eight U.S. landfill operators who are located near wind farms that are fifteen years of age or older, the estimated cost to put blade material in landfills, not including pretreatment and transportation costs, is approximately US$60 per ton. In the United Kingdom, where landfilling organics is not yet prohibited, the active waste disposal cost (which includes plastics) is approximately US$130 per ton.[21]

Incineration of blades is another disposal method with potential for energy and/or material recovery. Incineration of thermoset composites has some advantages, such as saving space and the economic value of utilizing the resin as a heat generator while recovering the fibers for different applications. Given that glass fiber is incombustible, the calorific value of GFRPs will depend on the proportion of polymer.[22] Despite these apparent advantages, there are important environmental issues with this disposal method. Combustion of GFRP is especially problematic because it can produce toxic gases, smoke, and soot that can harm the environment and humans.[23] Carbon monoxide and formaldehyde have been reported as residue from thermal degradation of epoxy resin.[24,25] Another residue is carbon dioxide,[24,25] which poses concerns regarding greenhouse gas emissions. In addition, about 60% of the scrap remains as pollutant ash after the incineration process, some of which is sent to landfills, potentially contaminating the sites. Possible emission of hazardous flue gasses is also among the issues with incinerating wind turbine blades. This is due to problems in the flue gas cleaning steps caused by the small fractions of glass fiber and pollutant byproducts.[8]

Other thermal processing methods include pyrolysis and fluidized bed combustion (FBC) and are aimed at recovering both the reinforcement fibers and the resin in the composite but can also recover combustion heat through a waste-heat recovery system.[26] The pyrolysis process decomposes the organic material into low molecular weight substances by applying heat in the absence of oxygen under controlled conditions.[27] The degraded polymer (in the form of smaller molecules such as oil, gas, or solid char) can be used as an energy source in other processes, while the glass fiber is left intact for recovery.[26,27] FBC is a similar method that consists of mixing fuel and air in a specific proportion for obtaining combustion.[28] Both processes recover glass and carbon fibers with some strength degradation that can be used for applications with lower mechanical demands, such as thermal resistance insulation material, although with limited economic viability.[26–28] The FBC for glass fiber composites seems to need a minimum production capacity of 10,000 tons/year to be economically feasible.[26] The pyrolysis process for carbon FRP seems to better retain the mechanical properties of the fiber and produce less fiber strength degradation compared with the FBC,

but it also produces environmentally hazardous off-gases and residues, including carbon monoxide,[b] carbon dioxide, and methane.[29] A combined pyrolysis-gasification process for wind turbine blades developed in Denmark has not been commercialized because it is not cost-effective.[26] One key issue is that all these thermal processing techniques for wind turbine blades would also require fragmentation of the material into smaller pieces through mechanical processing before being fed into the reactors, increasing energy consumption and carbon dioxide emissions.

Mechanical processing is a relatively simpler disposal method that consists of cutting, shredding, and grinding the material to separate the fibers from resins, so it can be repurposed. This process is energy intensive and produces small fiber particles with poor mechanical properties that can only be used as filler reinforcement material in the cement or asphalt industries.[30] The low market prices for substitute materials, the cost of grinding machines, and the energy required to operate them limit the cost-effectiveness of using ground thermoset composite.[30] The dust emitted in the grinding process of FRP creates occupational health and safety risks for workers. Inhalation, as well as skin and eye contact can produce moderate irritation to mucous membranes, skin, eyes, and coughing.[31] Occupational exposure and prolonged inhalation of such particles have been found to produce alterations of the cellular and enzymatic components of the deep lung in humans, identified as acute alveolitis.[32] While exposure control technologies such as suction filtration, humidification of the cutting site, and encapsulation of the grinding process can minimize some of these negative impacts,[33] they can also further increase the cost and make mechanical processing even less cost-effective. More research is needed about the effectiveness of these technologies at minimizing impacts and about the respiratory disease hazards related to exposure to particles from FRP.

The last method is chemical degradation, which consists of first mechanically reducing the size of the blades, then degrading them using a chemical solution.[34] The organic portion can be used as a feedstock for other processes and the rest is reinforcement and filler material that can be repurposed. Some of the advantages of this approach are the potential for recovering the resin and the ability to preserve the mechanical properties of the reinforcing fiber. The chemicals are used to release the fiber from the resin and/or to eliminate damages from the fibers after recovery. Although no industrial-level chemical recycling of thermoset polymers has been done yet, some hazardous chemicals such as nitric acids and paraformaldehyde have been used in testing and development processes.[35,36] Occupational exposure to these chemicals can produce harmful respiratory diseases including potential nasal cancer, and dermal health effects.[37,38] Despite this approach's ability to maintain the mechanical property of materials, the use of these toxic and hazardous chemicals limits its attractiveness.

A more desirable situation for blade disposal would be to achieve the property of reworkability in thermoset composites. The property of reworkability

mainly characterizes "the ability [of the thermoset material] to break down under controlled conditions [based on chemical and thermal techniques]."[39] The process reduces the mechanical strength, making it easier to reshape, recycle, and repair thermoset-based structures such as wind turbine blades.[39,40] A relatively new class of material called vitrimers can be thermally processed in a liquid state without losing network integrity, which means that upon heating, a rearrangement of the chemical bonds occurs that enable material deformation, processing, and recycling.[40] A reworkable thermoset means that the industry would have the ability to permanently deform the blade material without (or with minimal) loss in properties, as compared with what can be achieved at the moment. It would also avoid some of the negative environmental consequences and occupational exposure, while allowing for complete reuse of the material at the end of the blade's useful life. It would provide a wide number of options for blade treatment, such as easier repair without the need to replace the damaged blade, reutilization, or deformation to be used in other applications.

An Argument for More Research and Policy Intervention

There is a need to prioritize the safe and sustainable disposal of wind turbine blades before the anticipated influx of thermoset composite structures require dismantling. The current stock of blades and the actual manufacturing process use thermoset composites as the primary material, imposing constraints in both blade manufacturing and the disposal processes. If the goal is to increase the sustainability of the wind industry, we should find ways to encourage the development of better technologies in design, manufacturing, operations, and maintenance and disposal of the blades. Alternatives to the low-cost landfilling are needed as a disposal method due to its negative environmental impacts and the opportunity cost of recovered material. Given the current economic and regulatory climate, landfilling of blades will likely continue until another disposal method becomes as economically attractive. Consequently, discouraging the landfilling of blades will likely need effective policy intervention.

Besides economic obstacles, environmental issues and potential occupational exposures of combustion, pyrolysis, FBC, and chemical and mechanical processing must be addressed before any of these methods are widely applied in the industry. More research is needed to fully understand the health and environmental implications of some of these methods to workers, nearby communities, and overall population. Some of these technologies are relatively new or have had limited use in processing wind turbine blade material. As a result, there is considerable uncertainty about the feasibility of these technologies to function as a practical alternative for more sustainable wind turbine blade disposal. Because blades have to be cut on site before being transported to any processing plant or

landfill, it is also important to understand workers' exposure to components of fiber-reinforced composites, as well as technologies and methods to minimize this potential hazard.

Addressing these issues is particularly important for the disposal of the already-installed wind turbine blades. Nevertheless, because more blades are being manufactured and installed every year, the urgency to design blades that are economically and environmentally sustainable will create greater opportunities for new solutions and technology to emerge. However, these technology developments will depend on the extent to which they are prioritized in the future. In contrast, as a near-term solution, mechanical recycling with material reutilization in cement production may become more attractive if occupational safety can be ensured by developing the necessary standards and technologies. There are inhalable dust control standards for particulates exposure regulated by the U.S. Occupational Safety and Health Administration.[41] Implementing the aforementioned technologies (suction filtration and humidification) can help the potential industry comply with such standards and make mechanical recycling a more preferable option. However, more research is needed to ensure that those safety measures are adequate and that they will not further increase costs. As mentioned earlier, however, the property of reworkability would represent the gold standard in blade disposal as it implies complete reutilization of the materials without the need to use hazardous chemicals or to destroy/degrade the fibers. More research is still needed, but efforts are being made in that direction, as some research projects are funded by the National Science Foundation and the U.S. DOE.[42,43] Increasing budgets of research centers and laboratories to support research on alternative disposal methods would be one form of policy intervention.

In the short term, for thermal, chemical, or mechanical processing to become more appealing to the plastics recycling industry in the United States it must become a more economically competitive option. The capital investment and labor requirements make recycling too costly compared with the end market values of repurposed composite materials without some form of government intervention. Furthermore, it can be argued that landfill operators are being subsidized as they do not internalize the negative environmental externalities generated by landfilling waste. Therefore, tax breaks and subsidies are one method to reduce the cost for emerging and existing recycling companies compared with landfilling. For instance, policy intervention to prohibit or to impose a tax on the landfilling of wind turbine blades could encourage the industry to look for alternative disposal methods. In the current U.S. political environment, however, it will be challenging for such laws to pass at the federal level. Another possibility would be individual states banning the landfilling of composites thermoset. However, it would only be effective if enough states do it so as to make it economically unfeasible for wind farms to transport the blades at the end of life from one state to another state where it is not banned. A more

effective scenario could be a regional approach in which groups of states pursue common strategies that establish disincentives on the landfilling of composites thermoset.

Few individuals and organizations recognize the problems inherently related to blade recyclability. This situation creates an obstacle for promoting policy interventions to solve these problems. As a result, manufacturers, wind farm operators, and advocates have largely ignored the issue, focusing efforts on promoting wind energy and addressing other issues such as negative impacts on wildlife and noise generation. The wind energy industry would likely be reluctant to support such regulations unless it was part of a broader initiative that expands wind energy incentives to offset potential negative economic consequences. For policy interventions to succeed at the federal level, a major public awareness campaign is needed to raise consciousness levels about the current blade manufacturing and disposal practices. However, a public awareness approach should be carefully framed to include the true costs and benefits of wind energy as compared with fossil fuel alternatives. Over time, these issues will get attention in the media and will join the existing list of public environmental and ecological concerns, which the industry has been constantly looking for ways to address.[44] To address existing concerns, the wind energy industry has begun siting power plants in areas with lower bird and bat population densities, placing turbines in areas with low prey density, and using different numbers, types, and sizes of turbines to reduce bird and bat fatalities. Others actions include reducing aeroacoustic noise from the turbines, and technologies such as bubble curtains, cushion blocks, temporary noise attenuation pile design, vibratory pile drivers, and/or press-in pile drivers.[44]

Directives regarding producer responsibility can also be an effective policy tool. The *Extended Producer Responsibility* within the European *End of Life Vehicle Directive* establishes, among other things, that producers should manufacture vehicles that allow reusing and recycling the materials (e.g., automotive vehicles disposed after 2015 should allow 95% recovery with minimum 85% recycling), as well as be responsible for the disposal of the vehicles.[45] In the United States there is no federal legislation governing extended producer responsibilities, and End of Life Vehicle directives have been limited to voluntary programs.[46] Cherrington et al.[20] discuss how introducing the directive to the wind energy sector and developing strategies for blade disposal at an early stage would enable sustainable recovering and recycling methods to be in place when needed. Tojo[47] argues that, at least in theory, requiring producers to assume the management costs of disposal should incentivize them to improve environmental performance of the product and encourage innovative solutions.

At the same time, a transition to new manufacturing processes is needed to allow harvesting of wind energy in a more sustainable manner. A 2014 report by Global Wind Network[48] about the competitiveness of the wind energy sector concluded that to enhance blade manufacturing competitiveness in the United

States, research and development initiatives to optimize materials, designs, and processes are needed. For example, enhancements can be made by utilizing less material and more environmentally sustainable resources, such as less toxic and nonfossil fuel-dependent chemicals, by increasing blade life through better design, and by applying technologies such as condition monitoring methods and remaining useful life prediction techniques. Improvements could also involve developing materials that allow recyclability and blade designs that maximize power output and minimize impacts on the ecosystem. Such manufacturing innovations will contribute to the long-term sustainability of the wind energy industry. Policy interventions would significantly aid this effort and could include encouraging global collaboration that increases knowledge transfer from learning-by-doing and learning-by-searching processes, increasing the budget allocated to wind energy research for the DOE, National Science Foundation, and other publicly funded departments and laboratories, as well as providing subsidies or tax credits to companies allocating a percentage of their revenue toward research and development.

Technological Innovation

Researchers have been investigating some technological innovations. One potential development would be the manufacturing of blades using thermoplastics composites instead of thermosets. Contrary to thermosets, thermoplastic materials soften when heated and do not cure or set, making them easier to recycle. Some effort is being made to substitute thermoset composites with thermoplastic composites in some components of offshore wind turbine blades,[49,50] but technical limitations exist. The higher viscosity of the melted thermoplastic means slower flow of material, which makes it difficult to manufacture large, utility-scale blades.[50] The thermoplastic has higher viscosity than the thermoset, but a special reactive thermoplastic that processes like a thermoset, and that can be used to prepare thermoplastics composite, will flow into the mold and solidify faster, reducing the processing time.[50] Manufacturing blades with thermoplastic can be faster with the injection molding process, which consists of injecting the thermoplastic with pressure to fill a mold. However, vacuum infusion, instead of injection molding, is used in blade manufacturing because it reduces the formation of voids in laminates of large areas. Another drawback of using thermoplastic is that, it requires higher processing temperature, increasing the manufacturing costs. In addition, mechanical properties such as static and fatigue strength of thermoplastic are less suitable for manufacturing wind turbine blades,[49] which are subjected to many different environments at the wind farms. While there are technical limitations, the use of thermoplastic composites is becoming attractive for blade manufacturers due to ease of repair, recyclability of the material, and the short mold-cycle times in manufacturing.[49,50] While those limitations prevent it from being the solution in the near future, the use

of thermoplastics would nevertheless constitute an important contribution to the recyclability of the blades.

While the use of thermoplastic would allow for blades recycling, it is not entirely environmentally sustainable because it may involve a petroleum-based resin. Therefore, another potential development in the manufacturing process of wind turbine blades is the substitution of petroleum-based thermosets with bio-based thermosets.[51,52] In particular, one novel research approach is focused on the use of thermoset epoxies that are easily produced from a vegetable oil, minimizing energy intensity and costs.[43] Some research indicates that epoxidized linseed oil can be the basis for a thermoset whose mechanical properties are comparable to petroleum-based epoxy resins.[51,52] Ongoing work aims to optimize the epoxidized linseed oil system for use in wind turbine blade manufacturing. In addition, current research suggests that another advantage to the use of epoxidized linseed oil is that it is much safer for workers than conventional epoxy resins based on bisphenol A and epichlorohydrin.[43] The production of epoxidized linseed oil is a relatively clean, efficient process, without toxic reagents or byproducts involved. It is also less reactive and more stable at room temperature, making it safer to use than conventional epoxies,[43] where the curing process is often so exothermic that it can actually cause fires if not properly managed.

The other part of the research is focused on achieving the property of reworkability in both bio-based and conventional epoxies,[43] which would represent another medium- to long-term solution for optimal disposal. An optimal disposal method for wind turbine blades would overcome some of the issues with current practices and would require the least amount of effort and energy to implement while maintaining most of the value of the material. Unlike thermoplastics, current thermoset-based composites are by definition not reworkable once their curing is complete. As noted earlier, the reworkability of thermoplastics makes them attractive for effective blade recycling due to the reduced economic, occupational health and environmental consequences versus current disposal methods of thermoset. Being able to induce some measure of thermoplastic-like reworkability in thermoset-based composites would enable the bending, warping, or reshaping of (segments of) used blades into other shapes for different purposes without a significant loss in properties.[43] In addition, it might help to improve the efficiency of the manufacturing process and reduce variability, defect concentrations, and the need to overdesign structures by enabling the automated production of highly uniform flat plates that could be shaped after the fact into more complex geometries.[43]

Conclusion

Finding better ways to manage the expected high number of blades in need of disposal is important in order to harvest wind energy in a truly sustainable

manner. Better management would mean that economic and societal needs for clean energy are fulfilled without compromising the environment. None of the current methods allow for optimal wind turbine blade disposal. All of them carry potential economic, environmental, and occupational health concerns. Policy interventions such as allocation of more research funding to blade manufacturing and disposal, the provision of incentive mechanisms to recycling, and directives of producer responsibility could help overcome or minimize some of the challenges associated with disposing of wind turbine blades. However, some of these policies are likely to be implemented only when environmentalists and the general public become aware of and understand the real extent of such challenges. We believe that the best option is to move toward a different, more sustainable manufacturing process in material and design that also allows for optimal disposal. That result could be achieved through greater government funding for research and development, as well as tax credits for companies which invest resources in research and development.

Some potential technological innovations include the use of thermoplastics instead of thermosets and the use of bio-derived resins instead of conventional, petroleum-based epoxy resins, in the manufacturing process of the blades. The former could have important implications for blade recyclability and costs. The latter promises a more sustainable manufacturing process using bio-based feedstocks such as vegetable oil. They both demonstrate considerable potential as more sustainable blade manufacturing and disposal processes. These initiatives, however, still must address important technical issues before they can be applied in utility-scale blade manufacturing. Realizing the property of reworkability in thermoset-based composites would allow for optimal disposal since it would allow for complete reutilization of all the materials in the blade.

If the industry cannot come up with more sustainable manufacturing and disposal processes, public acceptance of wind energy would decline if the public becomes aware of these issues, inhibiting its growth as one of the main sources of electricity generation in the United States. There is great potential in wind energy because it is economically viable and much more environmentally friendly than fossil fuel-based electricity generation. It has become cost-effective for the electricity generation industry and is cheaper than other renewables, such as solar. However, the inability to overcome the barriers to wind turbine blade manufacturing and the continued landfilling of blades will represent a real challenge for wind energy expansion within the U.S. energy portfolio in the future.

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Notes

a. A 20-meter blade would use 41.2 kg of balsa wood and would produce about 876,000 kWh per year.[18] That amount of wood would generate 0.0000534 tons of CO2-e in a landfill environment. In contrast, the amount of natural gas that would have to be burnt to produce the same amount of energy (876,000 kWh/year) would generate 0.3261 tons of CO2-e. This would be an underestimated difference given that bigger blades are more energy efficient (for details on the formula used, see http://www.environment.gov.au/system/files/resources/b24f8db4-e55a-4deb-a0b3-32cf763a5dab/files/national-greenhouse-accounts-factors-2014.pdf).

b. Carbon monoxide is not a greenhouse gas, but it should be considered environmentally harmful because it is "a pollutant that affects methane, carbon dioxide, and tropospheric (lower atmospheric) ozone," which "plays a role in both air pollution and climate change..." (see http://www.giss.nasa.gov/research/briefs/shindell_09/).

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Direct detection of *Coccidioides* from Arizona soils using CocciENV, a highly sensitive and specific real-time PCR assay

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Abstract

Coccidioides immitis and *Coccidioides posadasii* are soil fungi endemic to desert regions of the southwestern United States, and the causative agents of valley fever, or coccidioidomycosis. Studies have shown that the distribution of *Coccidioides* in soils is sporadic and cannot be explained by soil characteristics alone, suggesting that biotic and other abiotic factors should be examined. However, tools to reliably and robustly screen the large number of soils needed to investigate these potential associations have not been available. Thus, we developed a real-time polymerase chain reaction (PCR) assay for testing environmental samples by modifying CocciDx, an assay validated for testing clinical specimens to facilitate coccidioidomycosis diagnosis. For this study, we collected soil samples from previously established locations of *C. posadasii* in Arizona and new locations in fall 2013 and spring 2014, and screened the extracted DNA with the new assay known as CocciEnv. To verify the presence of *Coccidioides* in soil using an alternate method, we employed next generation amplicon sequencing targeting the ITS2 region. Results show our modified assay, CocciEnv, is a rapid and robust method for detecting *Coccidioides* DNA in complex environmental samples. The ability to test a large number of soils for the presence of *Coccidioides* is a much-needed tool in the understanding of the ecology of the organism and epidemiology of the disease and will greatly improve our understanding of this human pathogen.

Key words: Coccidioides, valley fever, soil sampling, molecular detection, real-time PCR.

Introduction

Coccidioides posadasii and *Coccidioides immitis* are species of soil fungi endemic to arid regions of the Americas, and both cause valley fever, or coccidioidomycosis, a potential threat to the health of residents of the arid West.[1–4] Our current knowledge suggests that in Arizona, Mexico, Texas,

and Central and South America, this disease is caused by *C. posadasii*, whereas in the Central Valley of California and as far north as eastern Washington State the disease is caused by *C. immitis*. [5,6] Valley fever starts with inhalation of *Coccidioides* conidia from the environment, yet very little is known about the prevalence of the organism and factors associated with high levels of *Coccidioides* in the environment. Previously, cultures of *C. posadasii*, obtained from soils in Tucson, Arizona, showed the distribution of *Coccidioides* is sporadic and not explained by soil characteristics alone, suggesting a potential role of biotic or other factors in the distribution of the organism in the environment. [7] As soil disturbance is highly correlated with coccidioidomycosis,[8] our knowledge of *Coccidioides* ecology must grow in order to protect public health.

Current culture-based methods of environmental *Coccidioides* detection rely on standard media plate methods to grow the fungus directly from soils, or passage of soil solutions in mice susceptible to coccidioidomycosis and retrieval of viable culture from infected tissue.[5,7,9–13] Both methods have limitations and are cost and labor intensive. Direct culture requires a large number of plates, which is a complicated task in the confines of a biosafety level 3 (BSL3) laboratory, and generally results in low yields of *Coccidioides*.[14] The rapid overgrowth of other fungi that outcompetes *Coccidioides* is frequently stated as the main drawback to this method.[7,14] Mouse passage requires the presence of infectious arthroconidia, which only form at certain times of the *Coccidioides* life cycle, resulting in low success rates. Additionally, this method detects only strains pathogenic to the mice. If nonpathogenic strains exist, mouse passage will not indicate the true distribution of the organism in the environment.

Molecular based assays have been proposed as useful methods to screen soils for the presence of *Coccidioides*.[5,7] Several groups have developed nested PCR applications, targeting the multi-copy internal transcribed spacer (ITS) region common to many fungal species.[14–16] The methodology employs amplification of a conserved region flanking the variable ITS and uses the PCR product as the template for a second, more stringent PCR targeting a *Coccidioides*-specific region, followed by sequencing of the final product. However, the resulting sequence is frequently found to have low or no homology to *Coccidioides*.[15] Additionally, PCR involving the manipulation and further amplification of amplicon DNA is prone to contamination and false positives.[17,18] To improve this process, we developed a TaqMan PCR assay that is highly sensitive and specific to *Coccidioides*. The assay, CocciEnv, is based on the CocciDx assay, which has been validated[19] and recently received FDA clearance (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/denovo.cfm?ID=DEN170041) as a coccidioidomycosis diagnostic assay.[19] CocciDx has been used for limited soil and air analyses[5,20]; however, additional investigation of the assay and the target for environmental application was needed, and we therefore conducted this study. The assay targets a repetitive region of DNA known only in the *Coccidioides* genus. This method employs a sensitive and specific amplification that can provide results in a few hours after extraction of DNA. In order to further increase assay sensitivity for soil microbe detection, we increased the number of the target alleles captured by the assay, based on newly sequenced isolates of *Coccidioides*, and validated its use for environmental screening. We propose this as a robust method to detect *Coccidioides* DNA in environmental samples and as an indispensable tool for understanding the ecology of this understudied pathogen.

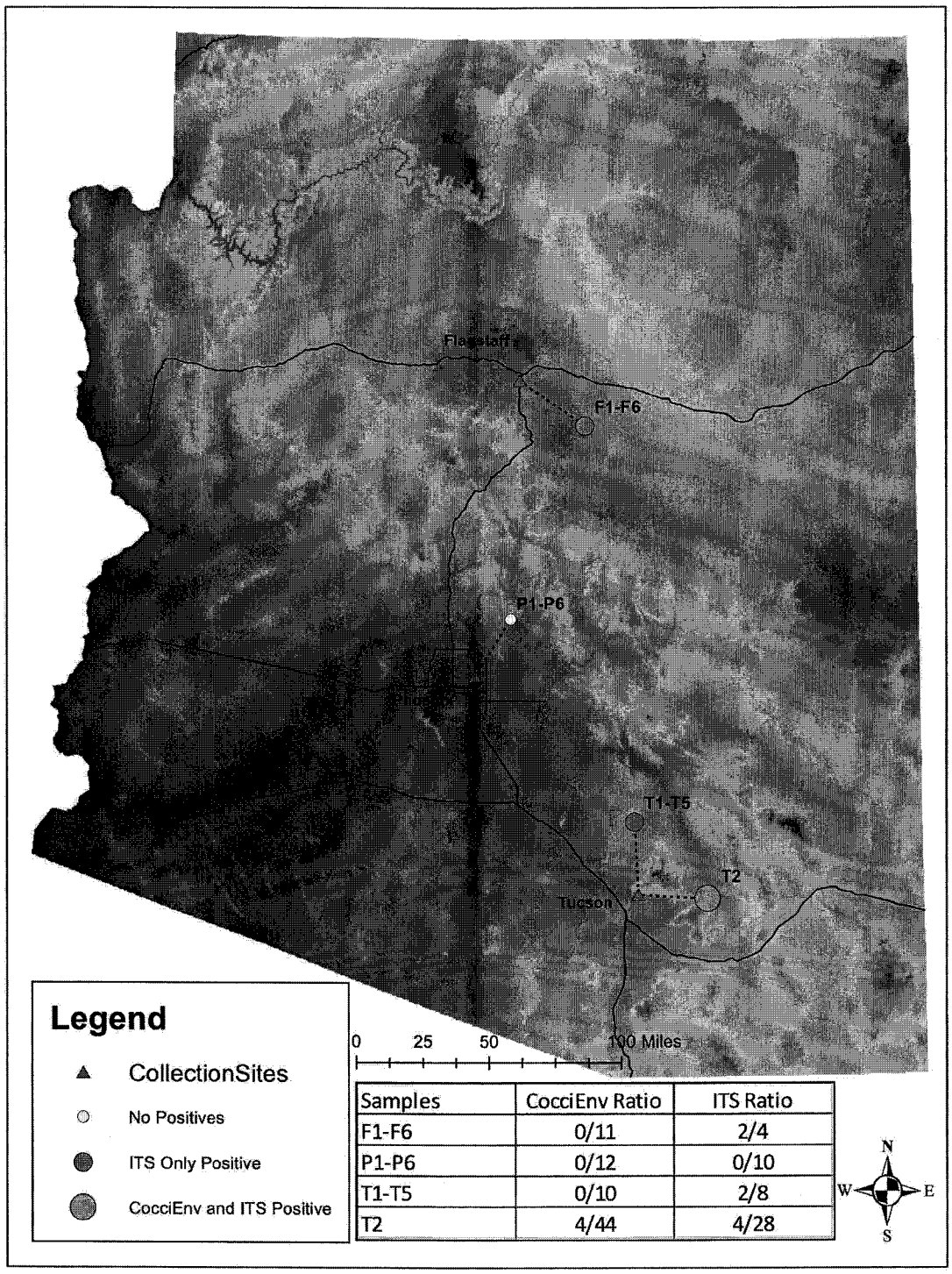
Methods

Site description and soil sampling

Soil sampling occurred in September–October, and the following April when the fungus is thought to be actively growing in the soil.[21–23] Several areas in Tucson that were previously identified as culture-positive for *Coccidioides*[7] were sampled as potential positive controls. Additional soil samples in the fall of 2013 were collected from rodent burrows in Phoenix and Flagstaff areas for comparison (Fig. 1, Table S1). Samples were collected from each site as a composite by removing the surface soil and collecting and combining 2 cm to 10 cm depth layers in sample collection bags or sterile 50 ml conical tubes. Implements were decontaminated with 10% bleach and rinsed with distilled water between collections, and samples placed in Ziploc gallon bags and surface-decontaminated with 10% bleach for transport on dry ice and storage at 4°C.

DNA extraction and preparation

For assay validation, genomic DNA from pure cultures of 562 *Coccidioides* collected from various clinical specimen types from several endemic regions was assayed (Table S2). All DNA samples were whole genome-amplified (WGA) using the REPLI-g Mini Kit (Qiagen, Boston, MA, USA) or illustra Single Cell GenomiPhi DNA Amplification Kit (GE Healthcare, Addison, IL, USA). WGA DNA was diluted 1:1000 before real-time PCR. Genomic DNA from four other Onygenales species, *Amauroascus mutatus* ATCC® 90275, *Amauroascus niger* ATCC® 22339, *Byssoonygena ceratinophila* ATCC® 64724, and *Chrysosporium queenslandicum* ATCC® 4404 was included in a set of genomic DNA from various fungal and bacterial species for



Samples	CocciEnv Ratio	ITS Ratio
F1-F6	0/11	2/4
P1-P6	0/12	0/10
T1-T5	0/10	2/8
T2	4/44	4/28

Figure 1. Map of Arizona sampling locations. Triangles represent sampling locations. Small yellow circles indicate samples collected from the site were negative for the presence of *Coccidioides* DNA. Medium red circles indicate that sites were positive using amplicon sequencing. Large gray circles indicate samples were positive using both methods.

specificity screening (Table S3). DNA was extracted from the latter isolates using the DNeasy Blood and Tissue Kit (Qiagen) with lytic enzymes appropriate for the species.

For soil samples, cell lysis and DNA extraction was conducted using the PowerSoil® DNA Isolation Kit (MO BIO). For each soil sample, DNA was separately extracted from 1 g of soil taken from two to four different sections of the collection bag to test reproducibility. (These replicates are labeled A, B, C, or D in Table S1.). Extractions were carried out according to manufacturer's instructions, with

TABLE 1. CocciDx and CocciEnv real-time PCR assays.

Assay component	Name	Sequence	Final concentration in PCR (μM)
CocciDx Assay			
Forward primer	CocciDx_F1	GTGTTAGGTAGTCCAACTAGCACCT	0.6
Forward primer	CocciDx_F2	GTGTTAGGTAATCCAACCAGCACCT	0.6
Forward primer	CocciDx_F3	GTGTTAGGTAATCCAACTAGCACCT	0.6
Reverse primer	CocciDx_R1	CTGATGGAGGACTCGTATGCTTGT	0.6
Reverse primer	CocciDx_R2	CTGATGGAGGACTTGTACACTTGT	0.6
Reverse primer	CocciDx_R3	CTGATGGAGGAATTGTATGCTTGT	0.6
Reverse primer	CocciDx_R4	CTGATGGAGGACTTGTATGCTTGT	0.6
Taqman probe	CDxQ_FAM-MGB	6FAM-ACCCACATAGATTAGC-MGBNFQ	0.25
CocciEnv Assay			
Forward primer	CocciEnv_F1d1	CGTTGCACRGGGAGCACCT	0.375
Forward primer	CocciEnv_F2	AAGCTTTGGATCTTTGTGGCTCT	0.375
Forward primer	CocciEnv_F3	AATTGATCCATTGCAAGCACCT	0.25
Forward primer	CocciEnv_F4	AATCCAACCTTTGGAACTACACCT	0.25
Forward primer	CocciEnv_F5	TTTTCCGGTATGGACTAGCACCT	0.375
Forward primer	CocciEnv_F6d2	TGTTAGGTAATCYAACYAGCACCT	0.125
Forward primer	CocciEnv_F7d2	TRTTAGGTAATYCAACTAGCACCT	0.125
Forward primer	CocciEnv_F8d1	TGTTAGATAATCCAACYAGCACCT	0.125
Forward primer	CocciEnv_F9d2	GKTARGTAATCCAACTAGCACCT	0.125
Forward primer	CocciEnv_F10d2	TGTTAGGTARTCCAACTAGCAYCT	0.125
Forward primer	CocciEnv_F11d2	TGTTAGGTAATCCAACTMGCACYT	0.125
Reverse primer	CocciEnv_R1	GATGGAGGACTCTATATGCTTGT	0.375
Reverse primer	CocciEnv_R2	ATGGAGGACTCGTTATGCCTGT	0.375
Reverse primer	CocciEnv_R3	GGAGGACCCGTATGCTTGTGT	0.375
Reverse primer	CocciEnv_R4	TGCTAAATGATGGAGGGCTTGT	0.375
Reverse primer	CocciEnv_R5	GATGGAGGCTCGTATGCTTGT	0.375
Reverse primer	CocciEnv_R6	AAGGGGTTTGTGGTGAATCCTTA	0.375
Reverse primer	CocciEnv_R7	CAGAAAAATAGCCGTATGCTTGT	0.375
Reverse primer	CocciEnv_R8d2	TRATGGAGRACTTGTATGCTTGT	0.125
Reverse primer	CocciEnv_R9d1	TGATGGAGGACTCGTATGCYTGT	0.125
Reverse primer	CocciEnv_R10d2	TGATGGARRACTCATATGCTTGT	0.125
Reverse primer	CocciEnv_R11d2	TGATAGAGAACTTGTATRCTTRT	0.125
Reverse primer	CocciEnv_R12d2	TGATGAAGAACTTRTATRCTTGT	0.125
Reverse primer	CocciEnv_R13d2	TGATRRAGGACTTGTATGCTTGT	0.125
Reverse primer	CocciEnv_R14	TGATGGAAAACTTGTATGCTTGT	0.125
Reverse primer	CocciEnv_R15d2	TGATGGAGGACTTGTAYAYTTGT	0.125
Reverse primer	CocciEnv_R16d2	TGATGGAGGACTTGTAYGCTTRT	0.125
Reverse primer	CocciEnv_R17d2	TGATGGAGGACTYATATGCTTRT	0.125
Reverse primer	CocciEnv_R18d2	GATGGAGGACTCGTWYGCTTGT	0.125
Taqman probe	CocciEnv_FMGB	6FAM-ACCCACATAGATTAGC-MGBNFQ	0.25

one exception: the FastPrep-24 Instrument at 6.5 m/s for 60 s (MP Biomedicals, Santa Ana, CA, USA) was used to bead-beat the sample. DNA was quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and diluted to a standard concentration of 100 ng/μl for PCR assays.

Real-time PCR assay development and screening

The real-time PCR assay, CocciDx (Table 1), was developed by the Translational Genomics Research Institute (TGen).[5] The CocciDx target was identified by surveying for repeat regions among *Coccidioides* genomes (http://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/coccidioides-genomes, sequence data now available as Genbank BioProject PRJNA46299. Repeat regions were first identified in the *C. immitis* RS genome by a pairwise BLAST of the genome against itself, using a word size of 50 and a minimum aligned length of 50 bp with 90% sequence identity, then determining which queries hit at multiple loci. These candidate repeat sequences were checked *in silico* for

ubiquity among *Coccidioides* genomes and for specificity to *Coccidioides* by BLAST of the NCBI nucleotide database. One candidate sequence was selected based on its high number of repeats, sensitivity, and specificity. In the NCBI database, the sequence is annotated as a copia-like retrotransposon. Alleles of the repeated region were aligned using SeqMan (DNAStar) and an assay was designed to conserved regions using Primer Express® 3.0 (ThermoFisher Scientific).

After *in silico* development, sensitivity, specificity, and limits of detection of the CocciDx assay were characterized.[19] The assay was optimized on the 7900HT Real-Time PCR System (ThermoFisher Scientific). Each 10 μl reaction mixture contained 1X PerfeCTa qPCR FastMix II (Quanta Biosciences, Beverly, MA, USA), assay concentrations outlined in Table 1, and 200 ng DNA template. Thermocycling conditions were initial denaturation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

To validate sensitivity, the CocciDx assay was screened across WGA DNA of 562 unique isolates of *Coccidioides*, including 40 *C. immitis*, 436 *C. posadasii*, and 86 *Coccidioides* species unknown (Table S2). To validate specificity, the assay was screened across a panel of DNA from various species including human, other fungal pathogens, one genetic neighbor, and several bacterial pathogens that may cause similar clinical presentation to coccidioidomycosis (Table S3). For limit of detection experiments a synthesized plasmid control containing one copy of the CocciDx target (Blue Heron Biotech, LLC, Tobermory, ON) was used. In order to precisely quantify copy numbers of the plasmid, serial dilutions of the plasmid, including dilutions down to extinction, were run on a real-time PCR assay that targets the β-lactamase gene present in the plasmids (Fig. S1). Using the Poisson distribution, plasmid copy number was calculated based on the observed number of amplification events of the lowest dilutions of the plasmid. To determine the limit of detection of CocciDx, 20 replicates of serial dilutions of the quantified plasmid control were screened to determine the lowest number of target copies that resulted in 95% positive results. The process was repeated in 60 replicates for confirmation.

With the recent deposition of new *Coccidioides* genome sequences in public databases,[24] we hypothesized that we could improve the analytical sensitivity of the assay by adding primers to capture more variants of the CocciDx target. Using a local BLAST database of the available *Coccidioides* genomes, we queried for hits with 100% identity to and 100% coverage of the CocciDx Taqman probe sequence. For each hit, we extracted the probe region and flanking sequence and aligned them. We designed 29 new primers to increase the number of alleles of the target captured by the assay (Table 1), and refer to the new environmental sample assay as CocciEnv. The total number of different alleles and copy numbers of the CocciEnv target in *Coccidioides* genomes were estimated bioinformatically (Fig. 2). The new assay was run using the same conditions as for CocciDx, with modifications only to primer concentrations (Table 1).

CocciEnv was subject to a more concise validation than for CocciDx given the extensive validation of CocciDx but included sensitivity and specificity screening across a subset of the DNAs mentioned above, along with DNA from four additional Onygenales species: *Amauroascus mutatus* ATCC® 90275, *Amauroascus niger* ATCC® 22339, *Byssoonygena ceratinophila* ATCC® 64724, and *Chrysosporium queenslandicum* ATCC® 4404 (Table S3). Additionally, CocciDx and CocciEnv were tested by using genomic DNA (not whole genome-amplified) from 23 *Coccidioides* isolates.

CocciEnv was run on soil DNA using the 7900HT Real-Time PCR System (ThermoFisher Scientific). Each 20 μl reaction contained 1 × PerfeCTa qPCR ToughMix (Quanta Biosciences) with 100 ng total DNA template and assay concentrations outlined in Table 1, with the following

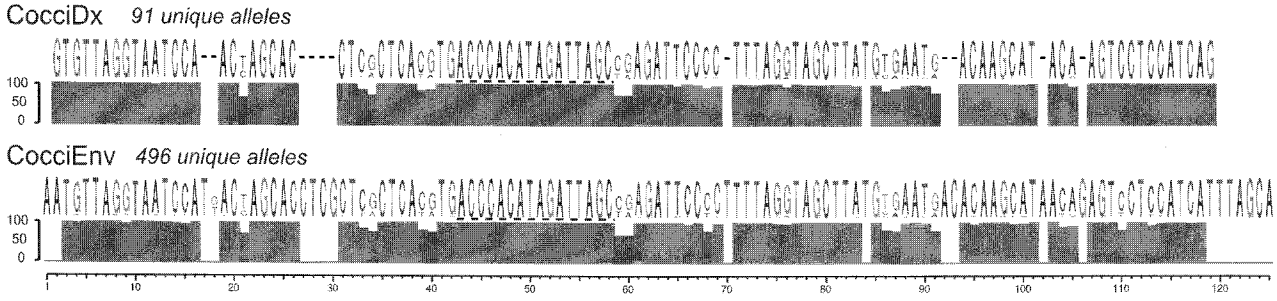


Figure 2. Consensus sequence comparison illustrating the allele diversity in Coccidioides genomes captured by CocciDx and CocciEnv. One representative sequence of each allele was included in the consensus (i.e., identical alleles were removed). The height of each nucleotide is proportional to its frequency in that position among the alleles. Gaps in the CocciDx consensus correspond to insertions in alleles captured by CocciEnv that are not captured by CocciDx. The histograms illustrate the percent frequency of each position in all alleles captured by each assay. The Taqman probe sequence is underlined with the dashed line. Figure was created using MegAlign Pro (DNAStar, Inc).

thermocycling conditions: initial denaturation for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were set up in a PCR cabinet to prevent contamination, and three technical replicates were run for each DNA sample. A reaction was considered positive if it showed logarithmic amplification, produced a C_T value of <40, and all controls performed as expected.

PCR validation of fungal genomic targets in soil

To confirm that soil DNA samples contained fungal DNA and were amenable to PCR, each was screened for fungal DNA using primers ITS1 and ITS4 targeting the ribosomal RNA operon.[25] Each 50 μl reaction contained 1 × MyFi™ Mix (Bioline) with 21 μl DNA template and 10 μM each forward and reverse primers, with the following thermocycling conditions: initial denaturation for 1 min at 95°C followed by 40 cycles of 15 s at 95°C, 20 s at 55°C, and 45 s at 72°C, and a final extension of 10 min at 72°C. PCR products were visualized via agarose gel electrophoresis. If bands were not present for a sample, it was not processed further, and DNA was re-extracted from that soil sample. Samples positive for fungal DNA were screened with CocciEnv, as well as by ITS2 amplicon sequencing (see below).

Validation of target amplification in soil DNA samples

As soil is a complex sample, Sanger sequencing was employed to confirm the presence of the assay target when detected in a soil sample. PCR was run in 20 μl reactions that included 100 nM of each primer CocciDx_F3 and CocciDx_R4 (Table 1), 2 ng DNA template, and *Taq* DNA polymerase (ThermoFisher Scientific). Thermocycling conditions consisted of an initial denaturation of 10 min at 95°C followed by 40 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C, and a final extension of 10 min at 72°C. PCR products were cleaned using ExoSAP-IT™ (Affymetrix, Santa Clara, CA, USA), and sequenced using the above PCR primers with BigDye® Terminator v3.1 chemistry (ThermoFisher Scientific). Reaction products were analyzed on a 3130xl automated genetic analyzer (ThermoFisher Scientific). Sequencing reaction results were assembled in Seqman (DNAStar).

ITS2 amplicon sequencing

The ITS2 region in fungal PCR-positive soil samples was amplified in triplicate using published primers.[26] PCR was performed in 8 μl reactions containing 0.1 U/μl Phusion Hot Start II DNA polymerase (ThermoFisher Scientific), 1 μM each primer, 200 μM dNTPs, and 6% glycerol (v/v). Thermocycling was as follows: 95°C for 2 min, and 25 cycles of 95°C for 30 s, 55°C for 30 s, 60°C for 4 min. Replicate reactions were pooled for indexing. Index PCR conditions and reagents were the same as above except for the indexing primers and 15 PCR cycles were performed. Indexed products were bead-purified,[27] quantified with PicoGreen® fluorescence (ThermoFisher Scientific), and pooled to equimolar concentrations. The final pool was bead-purified and quantified by qPCR using Library Quantification Kit, Illumina/ABI Prism (KAPA Biosystems) and sequenced in 2 × 250 mode on the MiSeq platform (Illumina).

We analyzed amplicon sequencing results using the TGen-developed bioinformatic tool, ASAP.[28,29] ASAP links together several bioinformatic programs with parameters set for customized sequencing analysis and results generation. In this case, ASAP first merged sequence read pairs with PEAR.[30] The reads were then trimmed of Illumina adapter (ligated during the sample library preparation process) and further trimmed based on sequence quality with Trimmomatic.[31] Specifically, a 5 bp sliding window across the read checked for average Phred scores below 20. Any windows that fit that criterion were removed. The full ITS2 *C. posadasii* reference sequence was obtained from the NCBI database (Genbank accession number KF539879) and trimmed to the expected amplicon size (334 bp) to serve as the reference sequence for the first round of ASAP. Trimmed, merged reads were then mapped to the reference sequence with the bowtie2 aligner.[32] Binary alignment map (BAM) files, generated by the aligner (one generated for each sample), were analyzed to determine the breadth and depth of coverage of the reference and identity to the reference. Thresholds to identify whether a sample was positive or negative were set at 100% breadth at 1 × depth of coverage at ≥97% identity (i.e., the full length of the 334 bp ITS2 reference sequence had to have a pair-merged read align with 10 or fewer single-nucleotide polymorphisms [SNPs]). This identity threshold was set according to the lowest identity of all known *Coccidioides* ITS2 sequences in the NCBI nucleotide database. Tablet[33] was used to verify results.

Upon analysis with ASAP, several samples were found for which reads aligned to the *C. posadasii* ITS2 amplicon reference that did not pass the 97% identity filter. To determine what other organisms might be the source of these sequences, any reads that aligned to the ITS2 reference that didn't meet the 97% identity, 100% breadth criteria were binned for analysis. A BLAST analysis of these reads showed hits to several other fungal species. These sequences were added as references for ASAP to determine if CocciEnv could be cross-reacting with other fungal species.

Results

CocciDx and CocciEnv assay validation and comparison

The WGA DNA samples from all 562 unique isolates of *Coccidioides* (Table S2) were positive on the CocciDx assay (real-time PCR Ct values were all < 35.0) and all DNA samples from various other species (Table S3) were negative (Ct values were all > 40.0), illustrating 100% sensitivity and 100% specificity. These data reflect the recently published CocciDx clinical validation data, in which sensitivity was 100% and specificity between 93.8% and 100% for DNA extracted from clinical specimens run on the GeneSTAT instrument (DxNA, LLC).[19] Using a serial dilution of a precisely quantified synthetic plasmid standard (Blue Heron Biotech, LLC), the CocciDx assay limit of detection was determined to be 15 target copies/reaction and the linear range was between 10^8 and 10^1 copies/reaction (Fig. S1).

On the CocciEnv assay, 45 out of the 45 *Coccidioides* WGA DNA samples tested were positive (Ct values < 35.0), and all 28 of the nontarget DNA samples, which included the Onygenales family members (Table S3), were negative (Ct value > 40.0). A comparison of CocciEnv and CocciDx showed that the CocciEnv assay resulted in an average of 1.8 (range of 1.6 to 2.1) Ct values earlier than those from the CocciDx assay when screened on the same genomic DNA, inferring a limit of detection three to over fourfold lower than that of CocciDx.

Genomes from 84 *Coccidioides* isolates were bioinformatically screened to determine the number of perfect matches to each assay that would result in the expected PCR product. Collectively in all 84 genomes, target alleles that were a perfect match to the CocciEnv assay were found a total of 4,614 times, which makes an average of ~55 copies/genome, while the alleles that were a perfect match to CocciDx were found 471 times, an average of ~6 copies/genome. Although the actual assays would likely capture additional alleles that are close, but not perfect, matches to the primer or probe sequences, thereby exhibiting sensitivity beyond what is described here, this was not further explored. The matches were dereplicated to determine the number of unique alleles that would be captured with a perfect match by each assay. CocciEnv captures 496 different alleles of the target, while CocciDx captures 91 different alleles (Fig. 2).

CocciEnv soil screening

Soil DNA was tested with CocciEnv in triplicate technical replicates. Results were considered positive if two of the three replicates had Ct values <40. Four samples tested positive out of 73 screened. These four samples were biological replicates of one soil sample collected near an apparently unoccupied large rodent burrow (Table S1), illustrating the reproducibility of both the DNA extraction and the CocciEnv assay.

ITS2 amplicon sequencing

Presumably, the vast majority of fungal species are not known, and soils have highly complex microorganism composition. We therefore set stringent parameters for determining the presence of *Coccidioides* in soil by ITS2 amplicon sequencing. The number of pair-merged reads that matched the *Coccidioides* ITS2 region reference sequence at ≥97% identity is shown in Table S1. Of the 50 soil samples tested, eight had one or more reads positive for *Coccidioides* (≥97% identity, Table S1, Table 2). Four of these are the four that tested positive by CocciEnv. Unfortunately, we did not have enough material to screen CocciEnv on three of the four other ITS2-positive samples (Table 2). The last sample had one read align and tested negative on CocciEnv.

We also identified by BLAST the closest species match to each pair-merged read that aligned to the *C. posadasii* ITS2 reference that did not pass the 97% identity threshold. The top BLAST hit for each was one of the following: *Chrysosporium keratinophilum*, *Chrysosporium tropicum*, *Aphanoascus verrucosus*, *Aphanoascus canadensis*, *Uncinocarpus reesi*, *Uncinocarpus queenslandicus*, *Arthroderma multifidum*, *Castanedomycs australiensis*, or *C. posadasii* (at <97% identity). The ITS2 sequences from these species were added to ASAP as references and results from this analysis are shown in Table S1. In 12 cases, the best hit of the reads was *C. posadasii*, at <97% identity, which could be indicative of an unknown *Coccidioides* ITS2 sequence or an unknown species. Seven of these 12 samples also had reads pass the 97% identity filter for *C. posadasii*, so were considered positive, suggesting the presence of unknown *Coccidioides* ITS2 sequences. Four of these seven tested positive on CocciEnv, while the other three were not tested. The five samples that did not have additional reads pass the identity filter tested negative on CocciEnv, suggesting the presence of a yet unknown fungal species (Table S1).

Discussion

Characterizing the natural reservoirs of *Coccidioides* is necessary for coccidioidomycosis epidemiology and public health protection. Unfortunately, a paucity of data exists to address this.[34] It is understood that *Coccidioides* has a sporadic, unpredictable distribution in the environment.[35] Because exposure of a susceptible host to arthroconidia often leads to infection, understanding the environmental

TABLE 2. Comparison of CocciEnv and ITS2 sequencing on a subset of soil samples. Data for all soil samples are in Table S1. 97% sequence identity is a common cutoff for species assignment for fungal metagenomics.

Sample ID	Location	CocciEnv mean C_t value	ITS2 Read counts ≥97% sequence identity	ITS2 Read counts <97% sequence identity
F2A	Flagstaff	Not performed	4	13
F2B	Flagstaff	Negative	0	0
F3A	Flagstaff	Negative	1	0
P2A	Phoenix	Negative	0	2
P2B	Phoenix	Not performed	0	0
P3B	Phoenix	Negative	0	0
P4A	Phoenix	Not performed	0	0
T2A	Tucson	Negative	0	11
T2B	Tucson	Negative	0	1
T3A	Tucson	Negative	0	10
T3B	Tucson	Negative	0	6
T4A	Tucson	Not performed	2	3
T4B	Tucson	Not performed	2	3
T5A	Tucson	Negative	0	4
T2-1a	Tucson	Negative	0	2
T2-1c	Tucson	Negative	0	2
T2-2a	Tucson	32.6	7	15
T2-2b	Tucson	31.3	14	22
T2-2c	Tucson	32.0	8	39
T2-2d	Tucson	32.0	22	43
T2-4a	Tucson	Negative	0	31
T2-4b	Tucson	Negative	0	12
T2-4c	Tucson	Negative	0	36
T2-4d	Tucson	Negative	0	7
T2-5a	Tucson	Negative	0	17
T2-5b	Tucson	Negative	0	10
T2-5c	Tucson	Negative	0	6
T2-5b	Tucson	Negative	0	13
T2-6a	Tucson	Negative	0	1
T2-10a	Tucson	Negative	0	12
T2-10b	Tucson	Negative	0	12
T2-10c	Tucson	Negative	0	11
T2-10d	Tucson	Negative	0	10

reservoir is critical to quantifying the risk of exposure. In fact, a recent study linked rising coccidioidomycosis cases with land use-induced soil disturbances in Antelope Valley in California.[8] With the development of a rapid, inexpensive, and high-performance screening tool, many ecological questions become answerable regarding favorable and unfavorable biotic and abiotic factors, mechanisms of dispersal, seasonality, and locations and persistence of *Coccidioides* foci in the environment.

Coccidioidomycosis is on the rise, and there are several nonexclusive phenomena that might be responsible; including population growth in endemic areas, increase of susceptible populations, heightened awareness of coccidioidomycosis, and increasing rates of exposure to arthroconidia through landscape disturbance.[8,36] Our understanding of the contributions of each of these factors is lacking.[36] A sensitive and specific soil-screening tool would enable studies to elucidate the role that landscape disturbance plays in the incidence of coccidioidomycosis. Additionally, such a screening tool would inform regulatory agencies in endemic regions (e.g., environmental, occupational health, corrections, and public health agencies) of risk of exposure to workers and communities within the vicinity of any proposed project where there is the potential for soil disturbance and dust, and inform remediation efforts. A recent epidemiological investigation of coccidioidomycosis outbreaks in prisons in California's Central Valley did not identify an association of coccidioidomycosis with outdoor activities.[37] Comprehensive soil surveys could pinpoint hotspots of *Coccidioides*, and be highly informative

for investigations such as this, and direct effective mitigation practices.

CocciEnv and its clinical diagnostic counterpart CocciDx are rapid, straightforward, highly sensitive, and inexpensive assays to detect *Coccidioides* DNA in environmental and clinical samples, respectively.[38] CocciDx recently received FDA clearance as a coccidioidomycosis diagnostic test (https://www.tgen.org/news/2017/december/06/tgen-technology-results-in-new-fast-accurate-valley-fever-test/). CocciEnv, with slight differences from CocciDx in primer number and sequence, is designed to be more specific and sensitive than CocciDx making it especially suited for testing environmental samples.

Of note, there were seven samples that contained several reads that matched the known *Coccidioides* ITS2 at ≥97% identity that also contained ITS2 sequences that did not pass the identity filter but whose top BLAST hits were *C. posadasii*. This may be evidence of a more genetically diverse population of *Coccidioides* than is currently described, or an as yet unknown *Coccidioides*-like fungus that cohabits with *Coccidioides*. The majority of sequences deposited in the NCBI database are clinical isolates, thus in-depth studies of *Coccidioides* soil isolates are necessary to determine if sequences detected might represent a non-pathogenic form of *Coccidioides*.

The identification of our highly-repeated assay target as a copia-like retrotransposon is not surprising. Retrotransposons replicate via RNA intermediates, which interact with a self-encoded integrase to integrate into the host genome, leaving the original template intact. In this way retrotransposons continuously increase in number. Eukaryotic genomes have numerous copies of some retrotransposons; the human genome contains more than a million copies of the *alu* retrotransposable element.[39] Thus, targeting a retrotransposon makes for a highly sensitive detection assay. We have targeted a portion of a retrotransposon in the Ty1/Copia superfamily, a superfamily originally defined in *Saccharomyces cerevisiae* and abundant throughout eukaryotic genomes.[40,41] The copia-like element we target with CocciEnv is specific to *Coccidioides*, making an ideal assay for applications requiring maximum sensitivity while maintaining specificity. Heterogeneity among copies of a given copia-like retrotransposon can be significant,[40] as we show here in *Coccidioides*, and is the reason behind the numerous primers in the CocciEnv assay.

The assay presented here addresses many of the limitations that have previously prevented needed fine-scale modeling of *Coccidioides* in the environment: the lack of a high throughput system to screen a large number of soils, the lack of sensitivity of microbiological methods, and the lack of reliable molecular tools. Additionally, CocciEnv obviates culture of *Coccidioides*, a BSL3 organism, and does not preclude the discovery of non-pathogenic strains, as mouse passage does. Our CocciEnv assay is sensitive and specific on the soils we tested, despite the fact that we did not find many positive soils. As the assay was designed using known fungal DNA sequence and validated with DNA from available fungal isolates, it is possible that we could detect unknown fungi resulting in false positive hits. Despite this, we promote the assay as a rapid and cost-effective screening tool to identify soils to more thoroughly investigate. For example, in the current study a majority of samples were negative for the two screening approaches, significantly reducing the number of samples that would be further processed using culture-based methods. Thus, the successful detection of *Coccidioides* with CocciEnv will be used as our screening method for future soil sample collections to identify novel sites for *Coccidioides* ecology research, recovery of viable organisms, and epidemiological information.

Supplementary material

Supplementary data are available at *MMYCOL* online.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Large-Scale Land Development, Fugitive Dust, and Increased Coccidioidomycosis Incidence in the Antelope Valley of California, 1999–2014

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Abstract Ongoing large-scale land development for renewable energy projects in the Antelope Valley, located in the Western Mojave Desert, has been blamed for increased fugitive dust emissions and coccidioidomycosis incidence among the general public in recent years. Soil samples were collected at six sites that were destined for solar farm construction and were analyzed for the presence of the soil-borne fungal pathogen *Coccidioides immitis* which is endemic to many areas of central and southern California. We used a modified culture-independent nested PCR approach to identify the pathogen in all soil samples and also compared the sampling sites in regard to soil physical and chemical parameters, degree of disturbance, and vegetation. Our results indicated the presence of *C. immitis* at four of the six sites, predominantly in non-disturbed soils of the Pond-Oban complex, which are characterized by an elevated pH and salt bush communities, but also in grassland characterized by different soil parameters and covered with native and non-native annuals. Overall, we were able to detect the pathogen in 40% of the soil samples ($n = 42$). Incidence of coccidioidomycosis in the Antelope Valley was positively correlated with land use and particulate matter in the air (PM10) (Pearson correlation coefficient >0.5). With the predicted population growth and ongoing large-scale disturbance of soil in the Antelope Valley in coming years, incidence of coccidioidomycosis will likely further increase if policy makers and land developers continue to ignore the risk of grading land without implementing long-term dust mitigation plans in Environmental Impact Reports.

Keywords Fugitive dust · Renewable energy · Coccidioidomycosis · Mojave Desert · Soil

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Introduction

Large-scale land development in the Antelope Valley, located in northern Los Angeles County in California, provides new residences for expanding populations, facilities for businesses, fields for agriculture, and

more recently provided opportunities for renewable energy production. However, arid and semiarid areas in the Southwestern US may require better care in managing soil disturbance from such projects because of greater risk of fugitive dust emissions and coccidioidomycosis, caused by the soil-born fungal pathogen *Coccidioides* spp. Fugitive dust is the suspension of particulate matter in the air by wind or human activities usually indicated as particulate matter up to 10 μm (PM10). The particulate matter is primarily soil but can contain crystalline silica, asbestos fibers, heavy metals, and airborne spores and conidia from microorganisms. Fugitive dust in general can cause breathing difficulties, low acute and chronic respiratory illnesses, increased risk of death from aggravated heart or lung disease [2, 12, 25, 27], increased risk of traffic accidents from poor road visibility [4], and reduced agricultural crop yield and desertification [75]. Fugitive dust emissions observed in the Antelope Valley frequently exceed California standards of 50 $\mu g/m^3$ for PM10 (24 h averages) and 30 $\mu g/m^3$ (annual arithmetic mean), respectively, which are stricter than federal standards (see http://www.arb.ca.gov/research/aaqs/caaqs/caaqs.htm for current California Ambient Air Quality Standards [CAAQS]). The increase in air pollution with coarse particulate matter (PM10) has raised the concern of public health officials and the general public [59], because of increased incidence of coccidioidomycosis among residents of the Antelope Valley (County of Los Angeles Department of Public Health, Annual Morbidity and Specials Studies Reports 2000–2014). Incidence of coccidioidomycosis in the Antelope Valley increased about 13-fold between 2000 and 2014 (supplementary figure S1). Strong Santa Ana winds can deliver dust from the desert to the LA Basin and deliver conidia of the pathogen to an area that is thought to be non-endemic for the pathogen [58].

The Antelope Valley is located in the Western Mojave Desert within the endemic zone of *Coccidioides* spp. which is comprised of certain areas in Arizona, California, Nevada, New Mexico, Texas, Utah, Washington, and Central and South America (see map in [55]). Fugitive dust that carries arthroconidia of *Coccidioides immitis* or *C. posadasii* can cause coccidioidomycosis in humans and animals primarily through inhalation of these dormant forms of the pathogen. Coccidioidomycosis primarily affects the pulmonary system in people and animals [16, 23], but dissemination of the disease to other organs can occur [28, 57]. Although about 60% of infected people develop mild to no symptoms, the other 40% experience weeks to months of debilitating disease that can include fatigue, shortness of breath, cough, fever, night sweats, loss of appetite or weight, chest pain, headache, body aches, skin rash, and pneumonia [63]. Less than 5% of these patients develop disseminated coccidioidomycosis, which increases the risk of lifelong complications and death [23, 43, 71]. Despite considerable efforts, no vaccine to protect humans from coccidioidomycosis currently exists [74].

The issue of fugitive dust carrying *Coccidioides* spp. arthroconidia is important not just for workers involved in land development projects, but also for residents of nearby communities, residents of newly built neighborhoods, and visitors working, studying, or travelling through the area. Furthermore, strong winds can transport conidia far distances, sometimes hundreds of miles, which can cause disease in humans and animals in non-endemic areas [21, 34, 35, 56].

The Antelope Valley of California provides an opportunity to examine how changes in the environment due to large-scale land development effect incidence of coccidioidomycosis in humans. Consisting of over 1800 mi^2 of fertile lands, the Antelope Valley is located approximately 2500 ft above sea level and is part of the "Lower Sonoran Lifezone" [53], sometimes referred to as the "High Desert," a common name for a subregion located mostly in northwestern San Bernardino County, northeastern Los Angeles County, and far eastern Kern County in areas above 2000 ft in altitude [39, 77]. The valley experiences an annual precipitation of 6–9 inches per year, a mean annual high temperature of 98 °F in the summer and 59 °F in the winter, with temperatures commonly above 100 °F in July and August [51]. Mountains along the Southern and Western border of the Mojave Desert block most of the moisture-bearing westerly winds from the coast, limiting precipitation and air humidity, and strong prevailing winds can result in severe dust storms [62].

The Antelope Valley has the greatest potential for land development in Los Angeles County, and its land use increased notably between 2001 and 2011 (Fig. 1). Guevara et al. [33] showed that disturbance of soil during the "housing boom" that peaked between 2004 and 2005 was positively correlated with a spike in coccidioidomycosis incidence at the same time. In

recent years, the Antelope Valley has become the focus of renewable energy projects to provide solar- and wind-generated energy for Southern California [11, 38]. Solar farms constructed by multiple companies will ultimately cover more than 30,000 acres in the valley (e.g. [17, 19, 31], for an overview of all planned renewable energy projects). Overall, the DRECP affects approximately 22,858,000 acres of semiarid and arid soils in the counties of Los Angeles, Kern, Inyo, San Bernardino, Riverside, Imperial, and San Diego.

Purpose and Scope

This project aimed to determine whether *C. immitis* is established in soils destined for photovoltaic system construction in the Antelope Valley, characterize the ecologic features of *C. immitis* positive sites, and correlate field findings with existing epidemiologic, geologic, and geographic data. Soil samples collected at six photovoltaic system sites either completed or destined for construction by 2014 or 2015 (Bureau of Land Management [BLM], CA, DRECP) were tested for the presence of *Coccidioides* spp. with a culture-independent polymerase chain reaction (PCR)-based approach. The sampled sites included non-disturbed locations covered with natural vegetation, predominantly *Atriplex polycarpa*; disturbed grassland with native and non-native annuals; fallow agricultural fields; and land impacted by sheep grazing. With this study, we hope to raise awareness of an increasing environmental health hazard that has been neglected in the past. Policy makers and others involved with large-scale land development projects could use the results from this study to implement better dust control approaches with more stringent requirements to reduce fugitive dust emissions and incidence of coccidioidomycosis and other dust-related illnesses among construction workers and the general public.

Materials and Methods

Soil Sampling Area

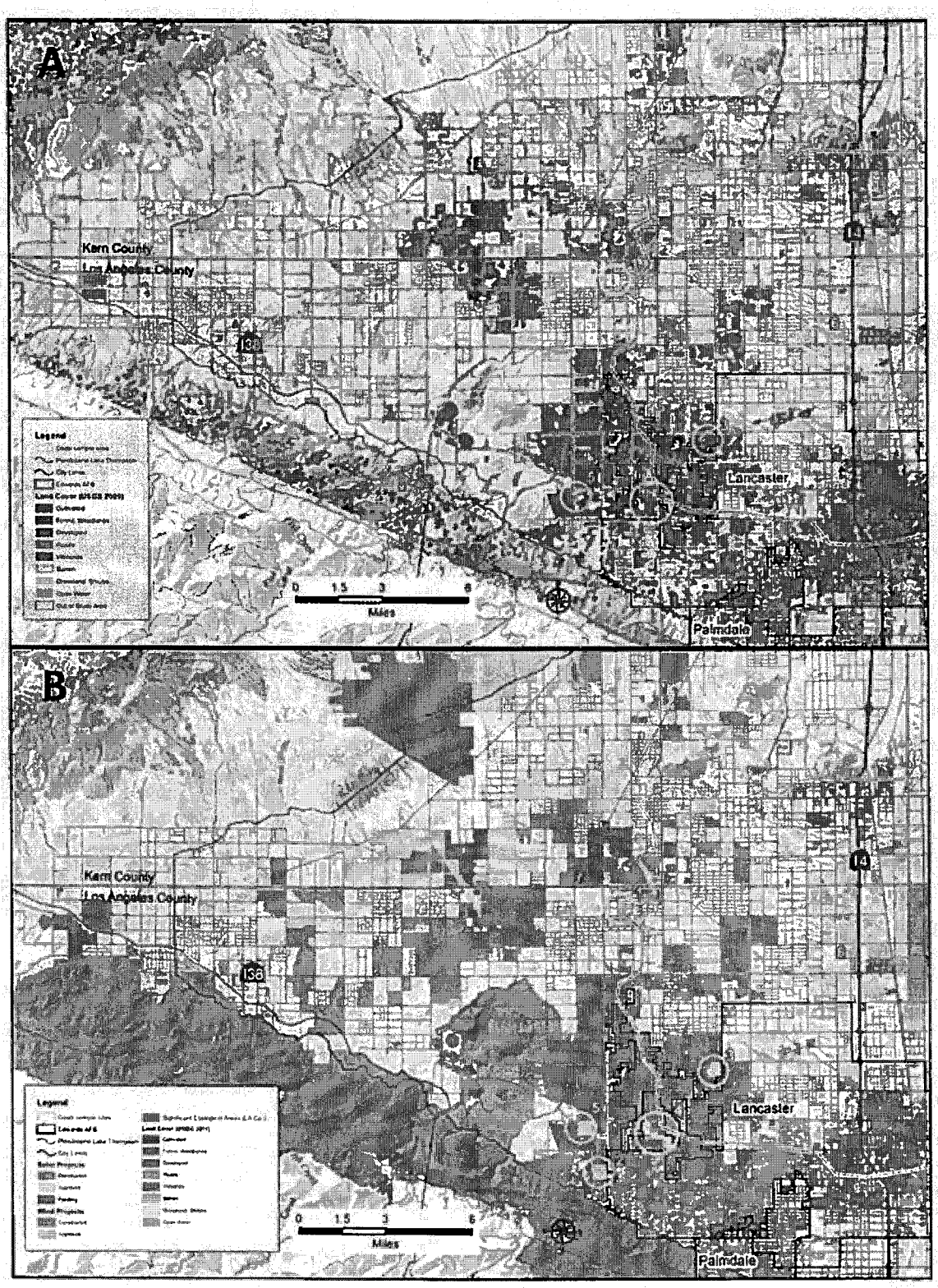
All soil sampling sites were located in the Antelope Valley subsection of the Western Mojave Desert in northern Los Angeles County west of the city of Lancaster and south of the rural town Antelope Acres (Fig. 2). The Antelope Valley watershed is a large topographic depression with no hydrologic outlet to the ocean. The runoff into the basin from surrounding creeks is conveyed via broad ephemeral washes toward several dry lakes. Two large dry basins, or playas, the Rosamond and Roger's dry lake beds (Kern County) form dominant natural landscape features within the Antelope Valley and are located east of the sampling area.

Ecological Landscape Characterization of Sampling Sites

Soil samples were collected at six sites destined for solar panel construction. These locations included site 1: North Lancaster Silverado Project, site 2: West Antelope Silverado Project, site 3: American Silverado Project, site 4A and B: Antelope Silverado Project, site 5: Silver Sun Silverado Project, and site 6: Lancaster WAD Project. Soil parameter information for all sites was obtained from the United States Department of Agriculture (USDA) websoilsurvey database. Coordinates of all sampling spots were documented, and the appearance of soils, as well as the vegetation cover (plant species and degree of coverage and disturbance), was documented. Plant species were identified using the Jepson Desert Manual [5] and other literature [49, 54]. Rodent activity was observed at all sites in form of pellets, burrows or both. Soil samples were collected from soil types that were dominant in the locations destined for solar panel constructions and were collected from 5 to 7 cm depth. The pH of all soil samples was analyzed as well (two replicates). Pictures of all sampling sites can be seen in Fig. 3. Detailed site descriptions can be found in supplementary table S1.

Soil Samples Collection

Thirty-one samples were collected at six sites on May 14 and 16 2014. Three to six individual soil samples (~25 g) were collected aseptically at several individual sampling spots at each of the six locations, using a small garden shovel and 50-ml Falcon tubes. After evaluation of all results from the 2014 sampling set, additional 11 soil samples were collected in May 2016 from site 6 only. All samples were transported to the laboratory on ice to prevent changes in the microbial communities and were stored at −20 °C before being


A
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◀Fig. 1 Overview of land use in the Antelope Valley in 2001 (a) compared to 2011 with indication of renewable energy projects (b). The six sampling sites investigated in this study are indicated as *yellow circles*. (Color figure online)

processed the following week. The sampling sites were documented photographically, coordinates were determined, and vegetation cover and visual appearance of all soils in regard to disturbance, erosion, rodent activity, soil moisture, and soil color was described.

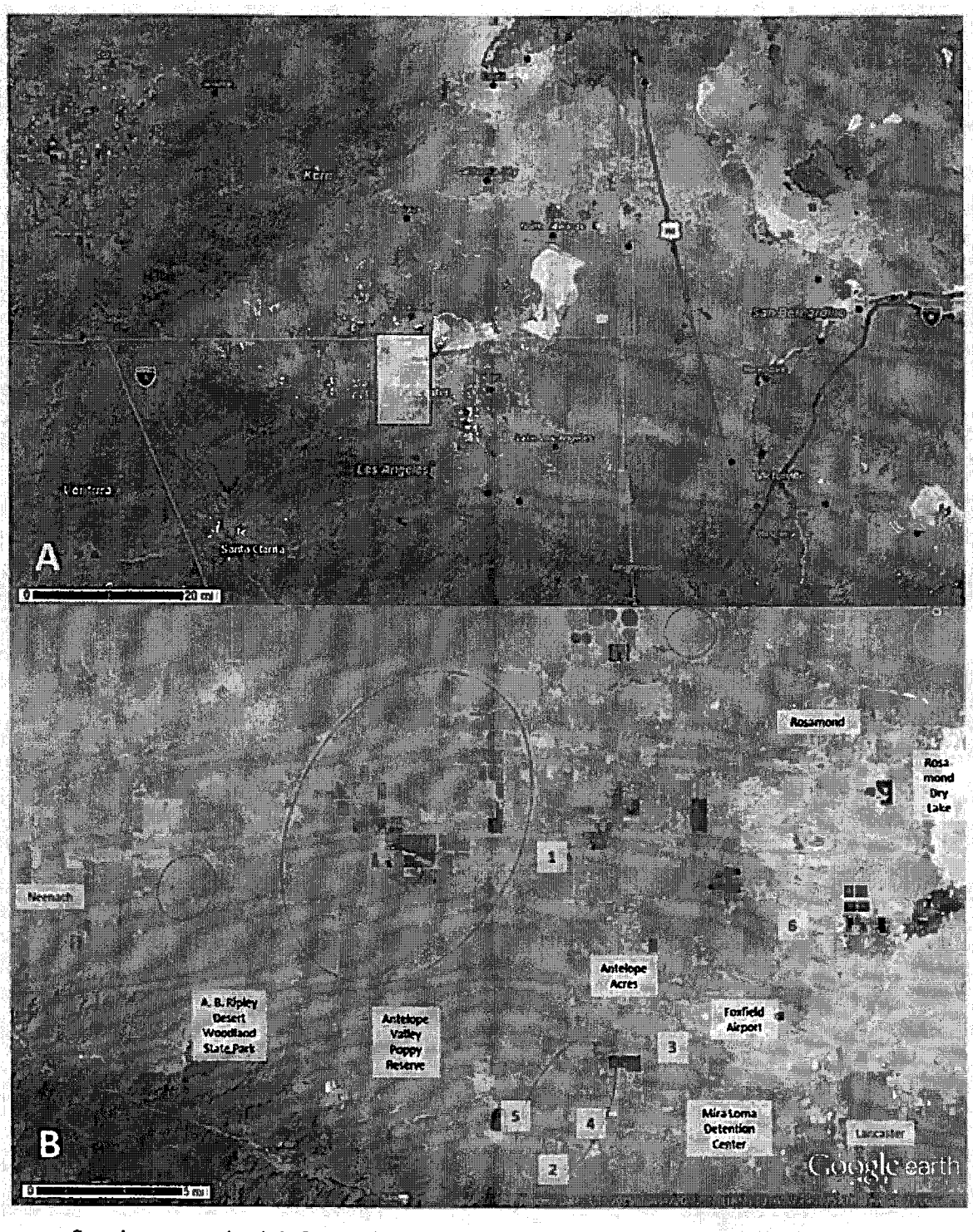
DNA Extraction and PCR

Soil samples were first mixed thoroughly by vortexing until homogenized. Prior to DNA extraction using the Powersoil DNA extraction kit (MoBio, Carlsbad, CA), 0.25 g of each soil sample was transferred into buffer-containing MoBio Powerbead tubes and incubated at 70 °C for 30 min, followed by an incubation step with 100 µl proteinase K (10 mg/ml) at 56 °C for additional 30 min [79] to enhance lysis of microbial spores and conidia. DNA extraction was performed according to the manufacturer's protocol (MoBio, Carlsbad, CA) using a MoBio vortex adapter for the bead-beating process. Two replicates were analyzed for each sample. The amount of DNA was quantified using the Qubit™ 3.0 Fluorometer (Invitrogen Life Technologies, Carlsbad, CA).

To determine the presence of *C. immitis* in all soil samples, a nested PCR approach based on the method published by Baptista-Rosas et al. [7] was used with modifications. A nested PCR can be superior to a one-step PCR method in that it excludes non-target DNA, therefore reducing possibilities of non-specific amplification. As the final diagnostic PCR step, we used 3 different primer pairs: (1) We replaced the originally suggested diagnostic primer pair with the ITSC1Af/ITSC2r primer pair (~220 bp, ITS 2 region) published by Greene et al. [32], which we found superior in specificity for *Coccidioides* spp. than the diagnostic primer pair used in Baptista-Rosas et al. [7] (data not shown), which was originally published by Binnicker et al. [9]. (2) We also used the EC3f/EC100r diagnostic primer set [36, 37] to detect *C. immitis*, which amplifies a ~500-bp amplicon, large enough to distinguish the 2 species within the genus *Coccidioides* and which covers both ITS regions of the ribosomal gene. (3) We also used the diagnostic primer pair ITS1Cf/ITS1Cr which amplifies a ~130 bp region of the ITS1 region of the ribosomal gene, published by Vargas-Gastélum et al. [76]. Overall, three sets of primers were used for each nested PCR approach. Aliquots of all PCR amplicons were analyzed using 2% (wt/vol) agarose gel electrophoresis to determine the correct size of the amplicons using a PCR marker (Promega G3161) (Promega Madison, WI) and ethidium bromide staining (0.5 mg/l). The first primer combination NSA3/NLC2 targets the ribosomal gene (18S and 5.8S DNA and both ITS regions) of all fungi and results in a ~1,100-bp amplicon. Amplicons from the NSA3/NLC2 combination were then used as a template in a nested PCR approach using primer combination NSI1/NLB4 which results in a ~910-bp fragment targeting a fragment of the ribosomal gene of Basidiomycetes and Ascomycetes only (see [7] for details). The final PCR step was the diagnostic PCR using a 1:25 dilution of the amplicons obtained with primer pair NSI1/NLB4 as a template and one of the diagnostic primer sets mentioned earlier in a final PCR. All PCR reactions were performed in duplicate, and the PCR cycling conditions as described in the original protocols were used (see Table 1 for details). PCR reactions contained 12.5 µl of GoTaq Green Mastermix (Promega, Madison, WI), 1.5 µl of each primer (10 pmol/µl), 2 µl of DNA extract or 1.5 µl of the product of a previous PCR reaction for the nested PCRs, as well as sterile water to a final volume of 25 µl. Negative control reactions, which contained all reactants with the exception of template DNA, were also included in all amplifications. These controls were carried through the entire nested PCR process along with the environmental products. Leftover PCR amplicons obtained via diagnostic PCR of approximately correct size (~220, ~500, ~130 bp) were subsequently treated with exoSAP-IT (Affymetrix, Santa Clara, CA), sequenced at the Center for Bioinformatics at the University of Florida, and subsequently compared to entries in the GenBank nucleotide database available at the National Center of Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) [1]. The sequencing step was necessary because occasionally false-positive amplicons were obtained.

Analysis of pH

Soil pH was determined on a 1:1 (w/v) soil/water mixture composed of 5 g of soil and 5 mL deionized



water. Samples were stirred before and after an equilibration period of 1 h and were then measured with an Oakton-510 bench-top pH meter (Oakton Instruments, Vernon Hills, IL) after calibration to pH buffers 4, 7 and 10. Two replicates were performed for each soil sample and the average was determined.

◀**Fig. 2** **a** Aerial view of the Western Mojave Desert with indication of our sampling area (*red rectangle*) in the Antelope Valley, west of the city of Lancaster (Los Angeles County). **b** Aerial photo of the Antelope Valley as of April 2015 (landsat 8). *Red numbers* indicate all sampling sites (*site 1* North Lancaster Silverado project, *site 2* West Antelope Silverado Project, *site 3* American Silverado Project, *site 4A and B* Antelope Silverado Project, *site 5* Silver Sun Silverado Project, *site 6* Lancaster WAD project). The *red circles* indicate areas where photovoltaic stations were constructed between 2009 and 2015. Construction sites outside these circles were not completed when this study was undertaken. The city of Lancaster is indicated in the *lower right corner* of the photo, south of the Rosamond dry lake bed. Also indicated are the Antelope Valley Poppy Reserve, the Arthur B. Ripley Desert Woodland State Park and the Mira Loma Detention Center. The settlement Antelope Acres is situated between the construction sites west of Foxfield Airport. (Color figure online)

Results

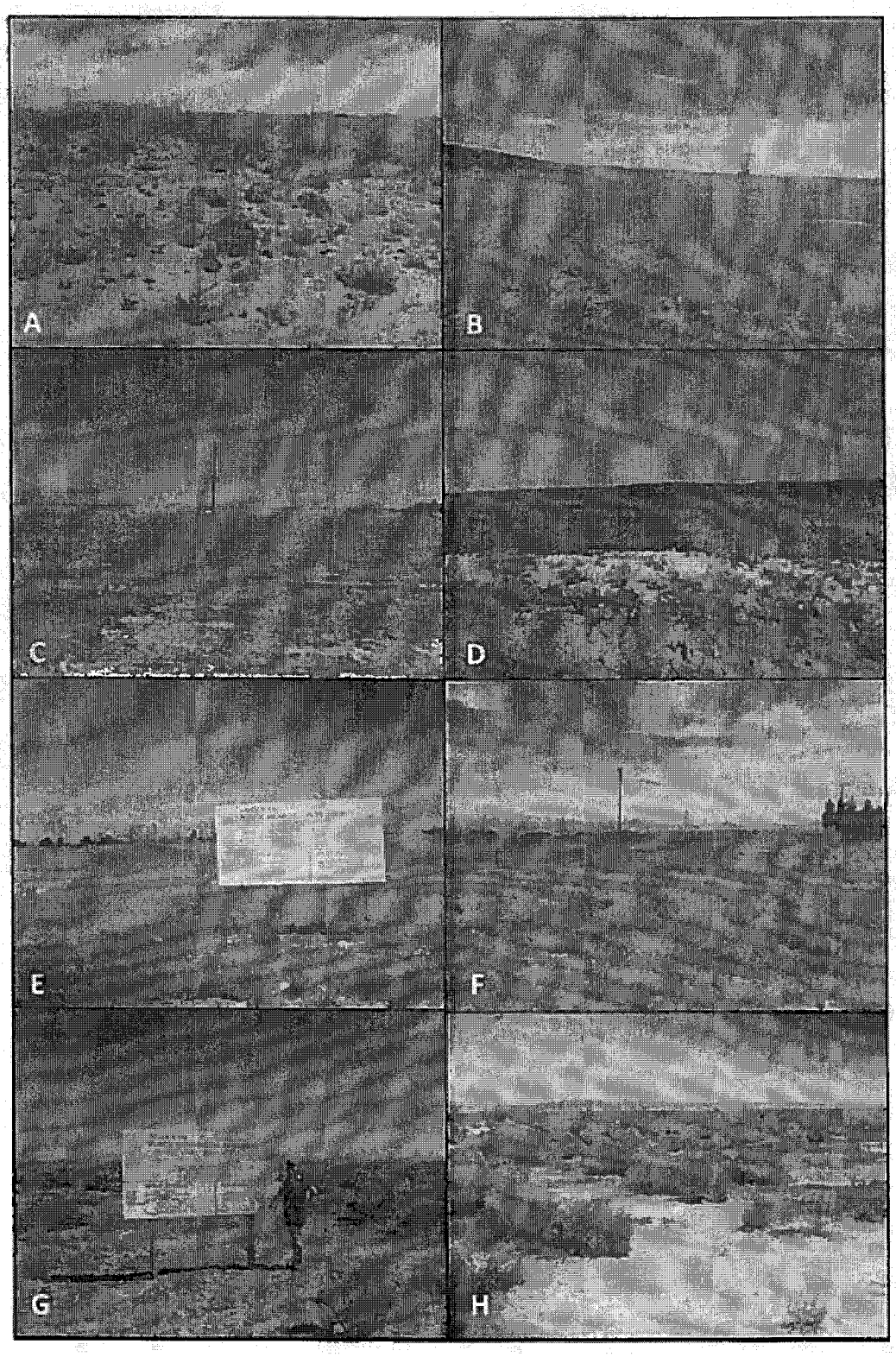
DNA Extraction and PCR

DNA of high quality was successfully extracted from all samples as confirmed by 2% agarose gel electrophoresis and subsequent ethidium bromide staining which resulted in distinct bands of non-sheared DNA. The amount of DNA extracted from 0.25 g of soil varied between soil samples and ranged between 29.2 and 9780 ng/ml. Site 6 had the smallest amount of DNA extracted (29.2–2420 ng/ml), whereas DNA extractions from samples collected at site 4 resulted in the highest amount of extracted DNA (3840–9780 ng/ml) (Table 2).

The nested PCR approach to detect *Coccidioides* spp. confirmed DNA of fungal origin in all soil samples and also confirmed DNA of Ascomycetes and/or Basidiomycetes in 90% of the samples. An example of nested PCR results including all three individual PCR steps with all diagnostic primer pairs is shown in Fig. 4 for a subset of samples. Table 2 summarizes the results of all PCRs and includes the closest matches in the GenBank nucleotide database for all sequenced amplicons. After comparing all sequences to entries in the Genbank nucleotide database, 17 soil samples (40.48%) were found positive for the pathogen with PCR amplicons of 99 or 100% similarity to a *C. immitis* entry in the GenBank nucleotide database (6.7, collected in 2016, showed a faint band of correct size with primer pair ITS1Cf/ITS1Cr which could not be confirmed by sequencing). Two additional soil samples resulted in amplicons that were 89% related to *C. posadasii* (sites 4B3 and 5.4 collected in 2014). Most of the false-positive PCR products were related to fungi in the order Capnodiales (*Cladosporium* spp.). In some occasions multiple species contributed to an amplicon, resulting in a "noisy" sequence that could not be identified. PCR products obtained with diagnostic primer pair EC3f/EC100r resulted more often in false-positive results than PCR products obtained with primer pair ITSC1Af/ITSC2r. Diagnostic primer pair ITS1Cf/ITS1Cr was the most specific of all three primer pairs tested, resulting in no false-positive amplicons. This primer pair was also the most sensitive one, because it detected the pathogen in 23.81% of the samples (28.57% if the two unconfirmed samples are considered as well). Primer pair EC3f/EC100r detected the pathogen in 11.9% of the samples, while primer pair ITSC1Af/ITSC2r detected *C. immitis* in 19.05% of the samples. Samples 6.2 collected in 2016 was the only sample that tested positive for the pathogen with all three diagnostic primer pairs. Five samples collected in 2014 and one sample collected in 2016 were indicated positive with two out of the three diagnostic primer pairs. Individual sampling spots where the pathogen was detected are shown in supplementary figure S2. Examples of high-quality sequences obtained with all 3 diagnostic primer pairs were deposited in the GenBank nucleotide database available at the National Center for Bioinformatics and Information (NCBI) (Accession No. KY306689-KY306699).

Characterization of Soil Samples

Variation in soil characteristics was observed for all sampling sites (USGS Soil Survey Antelope Valley, www.usdawebsoilsurveydatabase; Table 3; and supplementary figure S3). Soils in the sampling area varied in soil parent material, and in regard to chemical and physical parameters, as indicated by different USDA soil map units. Overall, the soil types that were the most common in our sampling area were characterized as Hesperia fine sandy loam (~10% of the sampling area), Greenfield sandy loam (~18%), Cajon loamy sand (~5.5%), Pond loam (5%), Rosamond fine sandy loam (~4%), Sunrise sandy loam (~6.5%); several others each covered <4% of the study area. Soils belonging to the Pond-Oban complex covered a large area of the valley around Rosamond and Roger's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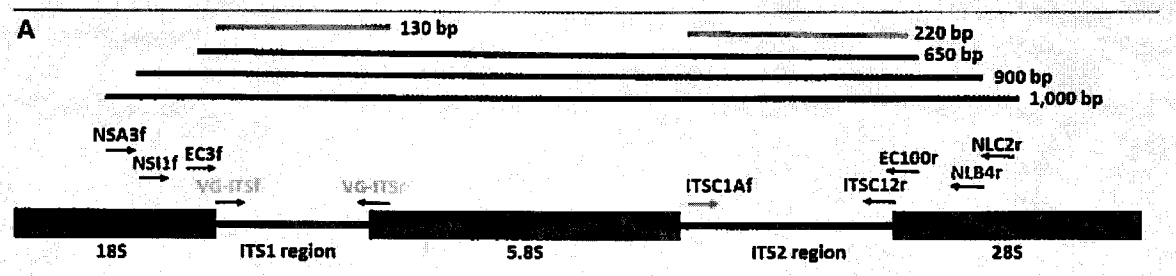
◀**Fig. 3** Landscape overview of all sampling sites at the time of sampling (May 2014). **a** Site 1, a disturbed site with scattered native and non-native vegetation. **b** Site 2, grassland with native and non-native annuals. **c** Site 3, disturbed land with scattered native and non-native vegetation. Surrounding areas grew rabbit brush (*Ericameria nauseosa*) (as can be seen in the *background*). **d** Site 4A, a disturbed area with scattered native and non-native vegetation. **e** Site 4B, grassland with native and non-native annuals. **f** Site 5, disturbed site with grasses and other non-native and native species. **g** and **h** Site 6, dominated by scattered salt bushes and occasional rabbit brush. Dried *Lastenia californica* can be seen in between the salt bushes (*Atriplex* spp.)

dry lake bed and comprised ~15% of the eastern study area where sampling site 6 was located. Soil pH generally increased with proximity to the Rosamond dry lake bed and ranged between pH 5 and 9.4. The pH varied considerably for subsamples from sites 2, 4A and 6, but were more uniform among samples from sites 1, 3, 4B and 5. Furthermore, average soil pH results observed in our laboratory differed from the averaged values indicated in the USDA websoilsurvey database. For example, fine sandy loam samples from sites 1 and 6 appeared less alkaline when analyzed in our laboratory. Samples positive for *C. immitis* also varied in pH, but the majority of the positive soil samples showed a pH higher than 7 and less DNA could be retrieved compared to samples with a lower pH (Fig. 5; supplementary figure S4). Soil parameters that were indicative of the presence of *C. immitis* in previous research in the Southern San Joaquin Valley (Kern County) [44, 45] predicted site 6 in the Antelope Valley as a potential growth site of the pathogen (Table 3). However, sites 2, 3 and 5 where the pathogen was detected as well were not indicated as potential growth sites based on soil parameters. Other soil types near our sampling sites, such as Rosamond loam and Tray loam, share some of the parameters that were indicative of the presence of the pathogen in the San Joaquin Valley, but these soils were not

Table 1 Position of primers on ribosomal gene (A), all primer pairs used for nested PCR reactions with PCR amplification conditions and references (B)

A



B

Primer	Sequence	Tm	Cycling conditions* Annealing temperature (°C)	Temperature (°C)	Time (s)	Number of cycles	Expected product size	Reference
NSA3f	5'-AAACTCTGTCGTGCTGGGGATA-3'	58.1	55	95, 55, 72	30, 40, 40	35	~1100	Martin [50]
NLC2r	5'-GAGCTGCATTCCCAAACAACTC-3'	56.8	55				~1100	Martin [50]
NSI1f	5'-GATTGAATGGCTTAGTGAGG-3'	50.8	60	95, 60, 72	30, 40, 40	35	~910	Martin [50]
NLB4r	5'-GGATTCTCACCCTCTATGAC-3'	51.7	60				~910	Martin [50]
ITSC1Af	5'-CATCATAGCAAAAATCAAA-3'	45.3	53	94, 53, 72	60, 60, 60	45	~220	Greene et al. [32]
ITSC2r	5'-AGGCCCGTCCACACAAG-3'	58	53				~220	Greene et al. [32]
EC3f	5'-ATTAAAGTGGCGTCCGGCTG-3'	58.1	57	95, 57, 72	30, 40, 40	45	~500	Johnson et al. [36,37]
EC100r	5'-CGATGAAGTGATTTCCCATACA-3'	52.3	57				~500	Johnson et al. [36,37]
VG-ITSf	5'-GTGGCGTCCGGCTGCGCACCTCCCCCGCGG-3'	78.1	70	95, 70, 72	30, 40, 60	45	~130	Vargas-Gastélum et al. [76]
VG-ITSr	5'-GCGCAAGGCGGGCGATCCCCGGCAGCC-3'	76.2	70				~130	Vargas-Gastélum et al. [76]

f Forward primer, *r* reverse primer

* All samples were subjected to an initial melting step of 94 or 95 °C for 10 min and a final extension step of 72 °C for 10 min

Table 2 Results of nested PCRs with indication of closest matches in the GenBank nucleotide database for amplicons obtained with the diagnostic primer pairs ITSC1Af/ITSC2r, EC3f/EC100r and ITS1Cf/ITS1Cr

Sample ID	NSA3/NLC2	NSI1/NLB4	ITSC1Af/ITSC2r		EC3f/EC100r		ITS1Cf/ITS1Cr		DNA extracted	
2014	All fungi	*Ascomycetes*/*Basidiomycetes*	*Coccidioides* spp.	% Similarity to closest match in GenBank	*Coccidioides* spp.	% Similarity to closest match in GenBank	*Coccidioides* spp.	% Similarity to closest match in GenBank	(ng/ml)	pH
1.1	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 99%	Negative		2800	6.90
1.2	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 94%	Negative		2640	7.13
1.3	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 95%	Negative		1960	7.13
2A.1	Positive	Positive	Positive	*Coccidioides immitis*, HG380500, 100%	False positive	unc. *Capnodiales*, JF691038, 93%	Positive	*Coccidioides immitis*, KM679413, 100%	3320	5.95
2.2	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 94%	Negative		4360	7.28
2.3	Negative	Negative	Negative		Negative		Negative		7280	6.90
2.4	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 90%	Negative		1406	5.24
2.5	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 93%	Negative		1858	6.29
2.6	Positive	Positive	Positive	*Coccidioides immitis*, HG380500, 100%	Negative		Positive	*Coccidioides immitis*, KM679413, 100%	4700	7.12
3.1	Positive	Positive	Positive	*Coccidioides immitis*, HG380500, 99%	False positive	unc. *Capnodiales*, JF691038, 95%	Positive	*Coccidioides immitis*, KM679413, 100%	3520	6.73
3.2	Positive	Negative	Negative		?*	Multiple sequences***	Negative		3480	6.76
3.3	Positive	Positive	Negative		?	Multiple sequences	Negative		2500	6.03
3.4	Positive	Positive	False positive	unc. *Eurotiales*, HQ389458, 95%	False positive	unc. *Capnodiales*, JF691038, 99%	Negative		6620	6.79
3.5	Positive	Positive	Positive	*Coccidioides immitis*, KJ783449, 100%	Positive	*Coccidioides immitis*, KJ783449, 100%	Negative		4400	7.11
4A.1	Positive	Positive	False positive	unc. *Eurotiales*, HQ389458, 96%	False positive	unc. *Capnodiales*, JF691038, 94%	Negative		3840	6.81
4A.2	Positive	Positive	Smear**		False positive	unc. *Capnodiales*, JF691038, 96%	Negative		9780	6.50
4A.3	Positive	Positive	Negative		False positive	*Ascochyta* sp., KC959210, 85%	Negative		6980	5.59
4B.1	Positive	Negative	Smear		False positive	unc. *Capnodiales*, JF691038, 95%	Negative		4520	7.38
4B.2	Positive	Positive	False positive		False positive		Negative		5900	7.55

Table 2 continued

Sample ID	NSA3/ NLC2	NSI1/NLB4	ITSC1Af/ITSC2r		EC3f/EC100r		ITS1Cf/ITS1Cr		DNA extracted	
2014	All fungi	*Ascomycetes/ Basidiomycetes*	*Coccidioides* spp.	% Similarity to closest match in GenBank	*Coccidioides* spp.	% Similarity to closest match in GenBank	*Coccidioides* spp.	% Similarity to closest match in GenBank	(ng/ ml)	pH
				Trichophyton terrestre, LN714614, 96%		unc. *Capnodiales*, JF691038, 94%				
4B.3	Positive	Positive	False positive	*Coccidioides posadasii*, KF386150, 89%	False positive	unc. *Capnodiales*, JF691038, 94%	Negative		8020	7.36
5.1	Positive	Negative	Negative		False positive	unc. *Capnodiales*, JF691038, 94%	Negative		7040	6.67
5.2	Positive	Positive	False positive	*Cladosporium macrocarpum*, KC311478, 95%	False positive	unc. *Capnodiales*, JF691038, 92%	**Positive**	***Coccidioides immitis*, KM679413, 100%**	3760	6.44
5.4	Positive	Positive	False positive	no similarity found	False positive	*Coccidioides posadasii*, JX051631, 89%	Negative		8040	6.69
5.5	Positive	Positive	Negative		False positive	Multiple sequences	Negative		3280	6.75
5.6	Positive	Positive	Negative		False positive	unc. *Capnodiales*, JF691038, 93%	Negative		7120	6.50
6.1	Positive	Positive	?	multiple sequences	**Positive**	***Coccidioides immitis*, KJ783449, 99%**	Negative		1796	7.44
6.2	Positive	Positive	**Positive**	***Coccidioides immitis*, KJ783449, 99%**	Negative		Negative		1270	8.15
6.3	Positive	Positive	**Positive**	***Coccidioides immitis*, KJ783449, 99%**	Negative		Negative		2420	7.04
6.4	Positive	Positive	Smear		Negative		Negative		2080	7.00
6.5	Positive	Positive	**Positive**	***Coccidioides immitis*, KJ783449, 99%**	**Positive**	***Coccidioides immitis*, KJ783449, 99%**	Negative		174	9.45
6.6	Positive	Positive	Negative		Negative		**Positive**	***Coccidioides immitis*, KM679413, 100%**	29.2	8.79
2016										
6.1	Positive	Positive	Negative		**Positive**	No signal (faint PCR product)	**Positive**	***Coccidioides immitis*, KM679413, 98%**	120	8.12
6.2	Positive	Positive	**Positive**	***Coccidioides immitis*, KJ783449, 99%**	**Positive**	***Coccidioides immitis*, KJ783449, 94%**	**Positive**	***Coccidioides immitis*, KM679413, 100%**	85.8	7.38
6.3	Positive	Positive	Negative		**Positive**	***Coccidioides immitis*, KJ783449, 99%**	**Positive**	No signal, faint PCR product	53	6.91
6.4	Positive	Positive	Negative		False positive	Fungal endophyte, KT291114, 94%	**Positive**	***Coccidioides immitis*, KM679413, 100%**	1014	7.94
6.5	Positive	Positive	Negative		Negative		Negative		324	8.25
6.6	Positive	Positive	Negative		Negative		Negative		55.6	9.53

Table 2 continued

Sample ID	NSA3/NLC2	NSI1/NLB4	ITSC1Af/ITSC2r		EC3f/EC100r		ITS1Cf/ITS1Cr		DNA extracted	
2014	All fungi	*Ascomycetes/Basidiomycetes*	*Coccidioides* spp.	% Similarity to closest match in GenBank	*Coccidioides* spp.	% Similarity to closest match in GenBank	*Coccidioides* spp.	% Similarity to closest match in GenBank	(ng/ml)	pH
6.7	Positive	Positive	Negative		False positive	Fungal endophyte, KT291114, 96%	Positive	No signal, faint PCR product	964	7.85
6.8	Positive	Positive	Negative		Negative		Negative		210	6.84
6.9	Positive	Positive	Negative		False positive	No similarity found	Negative		458	7.55
6.1	Positive	Positive	Negative		False positive	No similarity found	Positive	*Coccidioides immitis*, KM679413, 100%	244	9.21
6.11	Positive	Positive	Negative		Negative		Positive	*Coccidioides immitis*, KM679413, 100%	766	7.31

Positive samples and their closest match in the GenBank database are presented in bold

investigated in this study (soils where the pathogen was detected are indicated as positive [bold]).

Environmental Parameters and Incidence of Coccidioidomycosis

Environmental parameters, such as fugitive dust emission (PM10), total annual precipitation (inches), and wind speed (gust max.), were obtained for the Mojave Air Basin for the years 2000–2015. In addition, we obtained land-use data (acres) [15] and coccidioidomycosis incidence data [14] for the same time period and area (Fig. 6). An increase in incidence of coccidioidomycosis over time can be seen with highest incidence in the Antelope Valley in 2005, 2011 and 2014, spiking shortly after years with increased soil disturbance due to the "housing boom" between 2003 and 2007 [33] and the renewable energy boom described in this study. Between 2005 and 2014, the number of approved permits for solar farms and wind parks increased with additional large-scale and small-scale projects pending permission. So far, more than 20,000 acres of land have been disturbed as of 2014 for renewable energy projects in the Antelope Valley and the surrounding foothills of the Tehachapi and San Bernardino Mountains [30, 31]. The acreage of field crops increased by 48% compared to the year 2000 and then steadily declined by 2014 reaching values close to those documented before 2008 (County of Los Angeles Crop and Livestock Report 2014). The correlation between incidence of coccidioidomycosis in the Antelope Valley and the amount of acres of land disturbed for renewable energy projects and amount of acres under agricultural management (field crops) between 2000 and 2014 was strong, as revealed by a correlation coefficient of $r^2 = 0.623$ (Pearson product-moment correlation coefficient) and $r^2 = 0.388$. The correlation between PM10 (Mojave Air Basin) and disease incidence was at best weak with a Pearson coefficient of 0.283 and an r^2 value of 0.0664 (see Fig. 6 for all correlation values). However, the correlation between PM10 and incidence of the disease was strong when only the years between 2009 and 2014 were considered (renewable energy boom), with a Pearson coefficient of 0.641 and an r^2 value of 0.411. To investigate these relationships in more detail, a multiple regression analysis was conducted (Table 4). This analysis shows that neither PM10 nor levels of precipitation appear to have had a

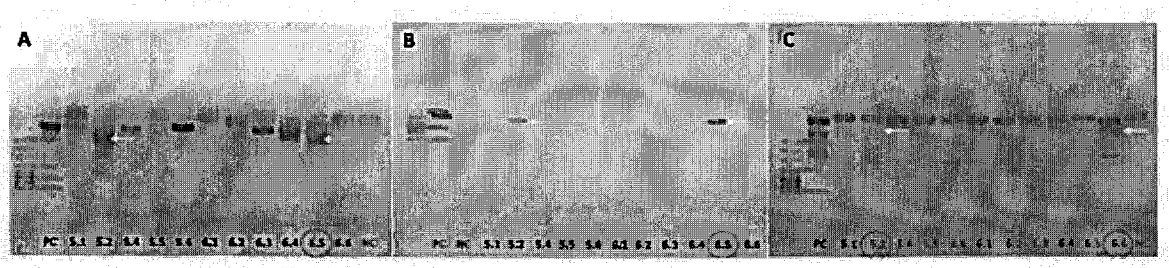


Fig. 4 Displayed are 2% agarose gels after ethidium bromide staining, showing examples of diagnostic PCR results for samples from some locations. Sequences from PCR amplicons circled in black were 99% related to a GenBank database entries of *C. immitis*. *White arrows* point toward amplicons of correct size including some that were revealed as false positives. **a** Results of amplification with primer pair ITSC1Af/ITSC2r showing amplicons of ~220. **b** Results of amplification with primer pair EC3f/EC100r showing amplicons of ~500 bp. **c** Results of amplification with diagnostic primer pair ITS1Cf/ITS1Cr showing positive results for samples 5.2 and 6.6 (2014). (*C.i. Coccidioides immitis* used as positive control; *NC* negative control)

significant relationship with coccidioidomycosis incidence (2000–2014). However, the total acres of land under the three land-use types considered (wind, solar and agricultural) did have a significant, positive relationship with coccidioidomycosis incidence ($p < 0.01$) over the same time period. We investigated the relationship with land use further by conducting a second multiple regression, removing the non-significant factors and disaggregating the three land-use types, to determine whether effects could be attributed to specific type of lands use. This analysis revealed no significant differences between the effects of the different land-use categories (Table 5).

Discussion

A correlation between soil disturbances due to large-scale renewable energy construction projects, agricultural management practices and PM10 fugitive dust emission with increased incidence of coccidioidomycosis was clearly indicated by results of this study. The increasing incidence of coccidioidomycosis in the Antelope Valley of California, which has reached epidemic character, is concerning and shown in supplementary figure S1. The *C. immitis* positive sites detected in this study are located west of the cities of Lancaster and Palmdale and south of the community of Antelope Acres which are part of what is known as the Greater Antelope Valley Economic Alliance (GAVEA) which has experienced a population increase of 24% between 2000 and 2010 (US Census Bureau). It has been predicted that the population will continue to grow another ~46% by 2035, to 758,881 residents [31]. The predicted population growth will result in continued urbanization as of yet unknown proportions, but certainly of significant size. Therefore, it is expected that fugitive dust emissions from ongoing construction sites will continue or even increase. This environmental health hazard will put humans and animals at an increased risk for contracting coccidioidomycosis, especially if dust mitigation continues to be inefficient or absent. In addition to increased urbanization and renewable energy development in this area, an ongoing drought with decreasing precipitation and sinking ground water tables has been blamed for soil erosion and fugitive dust development in the Antelope Valley. The ongoing drought has also resulted in a significant reduction in farming activities over the last years, resulting in large areas of abandoned fields. For example, the farmed acreage of orchards decreased from 2013 to 2014 by 53.06%, and the farmed acreage for grapes decreased by 22.6% during the same time in the County [48].

It has been difficult in the past to determine a clear correlation between incidence of coccidioidomycosis and certain environmental parameters, because of combined immediate or delayed positive or negative effects on the growth of the pathogen in the soil. Previous work by Talamantes et al. [72] determined a weak correlation between precipitation and wind speed and coccidioidomycosis incidence in Kern County. Smith et al. [69] and Kirkland and Fierer [40] had already pointed out that a rainy winter can cause an increase in coccidioidomycosis incidence in the following dry season, especially after a prolonged drought that might have caused a shift in the microbial community toward spore and conidia formers, among

Table 3 Averaged soil physical and chemical parameters for dominant soil types found in our sampling area with indication of soil map unit symbols, as obtained from the USDA websoilsurvey database (pH was also analyzed at CSUB). (Color figure online)

Soil parameters (averaged data)	Sampling sites				
Sites	1*	1, 3*	2*, 4A*, 4B*, 5	2, 4, 5*	6*
Map unit name	Rosamond fine Sandy loam	Hesperia fine Sandy loam	Greenfield Sandy loam	Ramona coarse Sandy loam	Pond Oban complex
Landform	Alluvial fans	Alluvial fans	terraces, alluvial fans	terraces	Basin floors
Parent material	Alluvium derived from granite	Alluvium derived from granite	Alluvium derived from granite	Alluvium derived from granite	Alluvium derived from granite
Map unit symbols	Ro	HkA	GsA/GsC	RcB/RcC	Pz
Physical parameters					
Surface texture	Fine sandy loam	Sandy loam	Sandy loam	Coarse sandy loam	Fine sandy loam
Clay (%)	18.8	13	11	7.5	25.7
Sand (%)	51.9	70.5	66	69.6	46.5
Silt (%)	29.3	16.5	23	22.9	27.8
Available water capacity (cm/cm)	0.14	0.13	0.13	0.1	0.08
Available water supply (0–25 cm)	3.45	2.5	3.25	2.5	2.04
Organic matter (%)	0.17	0.08	0.75	0.75	0.25
Water content (15 bar) (%)	12.1	8.1	7.4	6	15.8
Water content (1/3 bar) (%))	23.3	17.2	16.4	14.7	27.5
Sat. hydraulic conductivity (Ksat) (μm/s)	21.67	28	28	28	4.8
Chemical parameters					
pH (websoilsurvey database)	8	7.4	6.7	6.7	8.9
pH (determined at CSUB)	7.1 (site 1)	6.7 (site 3)	6.4 (site 2), 6.1/6.6 (site 4A/4B)	6.8	8
$CaCO_3$	5	2	0	0	0
Cation exchange capacity (CEC7) (milliequivalents/100 g)	10	7.5	7.5	7.5	15.8
Gypsum	0	0	0	0	0
Sodium adsorption ratio (SAR)	0	0	0	0	12.7
Electrical conductivity (EC) (decisiemens/m)	1.7	0.7	0	0	14
Wind erodibility index (tons per acre per year)	86	86	86	86	86
Detection of *C. immitis*	Negative	Positive	Positive	Positive	Positive

Soil parameters	Other dominant soils in the sampling area					
Sites	1	3	3	6	2	4
Map unit name	Rosamond loam	Cajon loamy sand	Tray sandy loam, Saline-alkali	Tray Loam	Hanford Coarse sandy loam	Sunrise sandy loam
Landform	Alluvial fans	Alluvial fans	Basin floors	Basin floors	Alluvial fans	basin floors
parent material	Alluvium derived from granite	Alluvium derived from granite	Alluvium derived from granite	Alluvium derived from granite	Alluvium derived from granite	alluvium derived from granite
Map unit symbols	Rp	CaA	Tv	Tw	HbC	Sv
Physical parameters						
Surface texture	Loam	Loamy sand	Sandy loam	Loam	Sandy loam	Sandy loam
Clay (%)	20.5	3.7	12.7	20	12.5	15
Sand (%)	34.7	83.1	65.8	42.1	68.2	65.9
Silt (%)	44.8	13.2	21.6	37.9	19.3	19.1
Available water capacity (cm/cm)	0.16	0.08	0.1	0.15	0.13	0.12
Available water supply (0–25 cm)	3.85	1.98	2.5	3.5	3.25	3
Organic matter (%)	0.17	0.57	0.58	0.75	0.58	0.25
Water content (15 bar) (%)	12.3	3.3	8.4	12.5	8.9	9.2
Water content (1/3 bar) (%))	27.7	10.9	17.7	27.1	17.8	18.3
Sat. hydraulic conductivity (Ksat) (μm/s)	9	92	21.7	9	28	28
Chemical parameters						
pH (websoilsurvey database)	8.2	7.2	9.1	9	6.7	7.9
pH (determined at CSUB)	Not determined					
$CaCO_3$	5	1	3	3	0	8
Cation Exchange Capacity (CEC7) (milliequivalents/100 g)	10	3	7.5	7.5	7.5	7.5
Gypsum	0	0	0	0	0	0
Sodium adsorption ratio (SAR)	0	0	3	3	0	0
Electrical conductivity (EC) (decisiemens/m)	1.3	0.2	5	5	0	1
Wind erodibility index (tons per acre per year)	56	134	86	48	86	86
Detection of *C. immitis*	Not investigated					

Soil parameters that were indicative of the presence of the pathogen in the Southern San Joaquin valley [44, 45] are indicated in red. Additionally, results from our PCR-based approach to detect *C. immitis* are included (at some sampling sites, more than one soil type was detected; therefore, the soil type of the soil samples analyzed is indicated with an *; soil types where the pathogen was detected are indicated with a red rectangle)

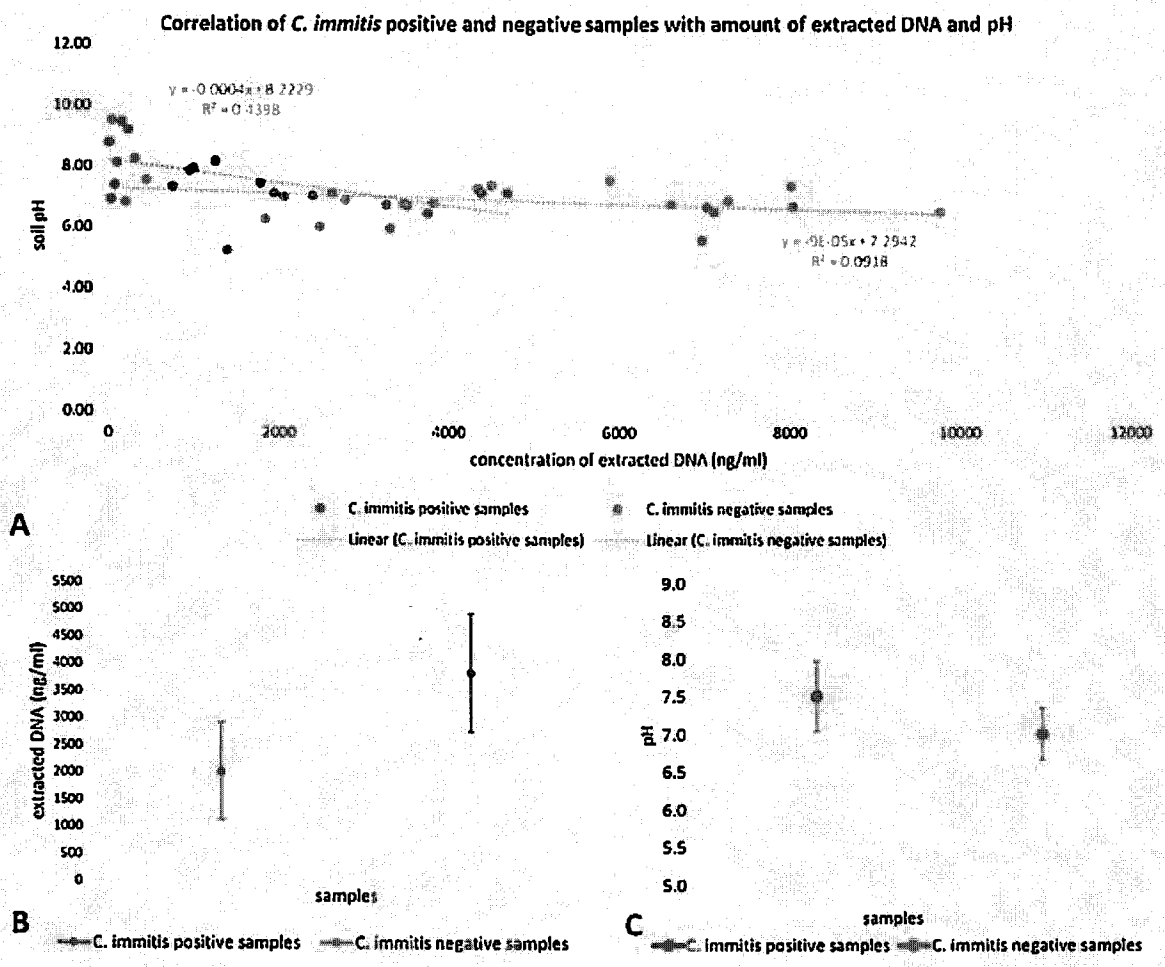


Fig. 5 a Correlation between soil pH and amount of extracted DNA. b The amount of extracted DNA from *C. immitis* positive soil samples was significantly lower than the amount of DNA extracted from *C. immitis* negative soil samples. c The pH of soils in which the pathogen was detected was higher than the pH of soils that were negative for the pathogen. However, the difference was not significant (data were normally distributed based on the Shapiro-Wilkes test for normality of the residuals). (Color figure online)

them *Coccidioides* spp. In our study, we were able to clearly link land use and soil disturbance to valley fever incidence, but also found a positive correlation between PM10 and wind speed; however, the correlation was rather weak. The continued increase in coccidioidomycosis incidence in 2012 and 2013 when construction of new renewable energy projects slowed down was likely due to the long-term effect of large areas of graded soils, which continue to be a major source of fugitive dust emission in the Antelope Valley and beyond. In the past, California had been plagued with long-term and short-term droughts, for example the prolonged drought from 1985 to 1992 which resulted in increased fugitive dust emissions that reached a 24-h record PM10 concentration of 780 $\mu g/m^3$ in downtown Lancaster in 1991 (Antelope Valley Air Quality Monitoring District).

We were able to detect the pathogen *C. immitis* predominantly in undisturbed alkaline soils of the Pond-Oban complex, located closest to the Rosamond dry lake bed, a location commonly referred to as "barren land" with different species of salt bushes, that indicate a saline and alkaline environment. Site 6 was the only sampling site that was suspected to harbor *C. immitis* based on averaged soil parameter information (USDA websoilsurvey database) that

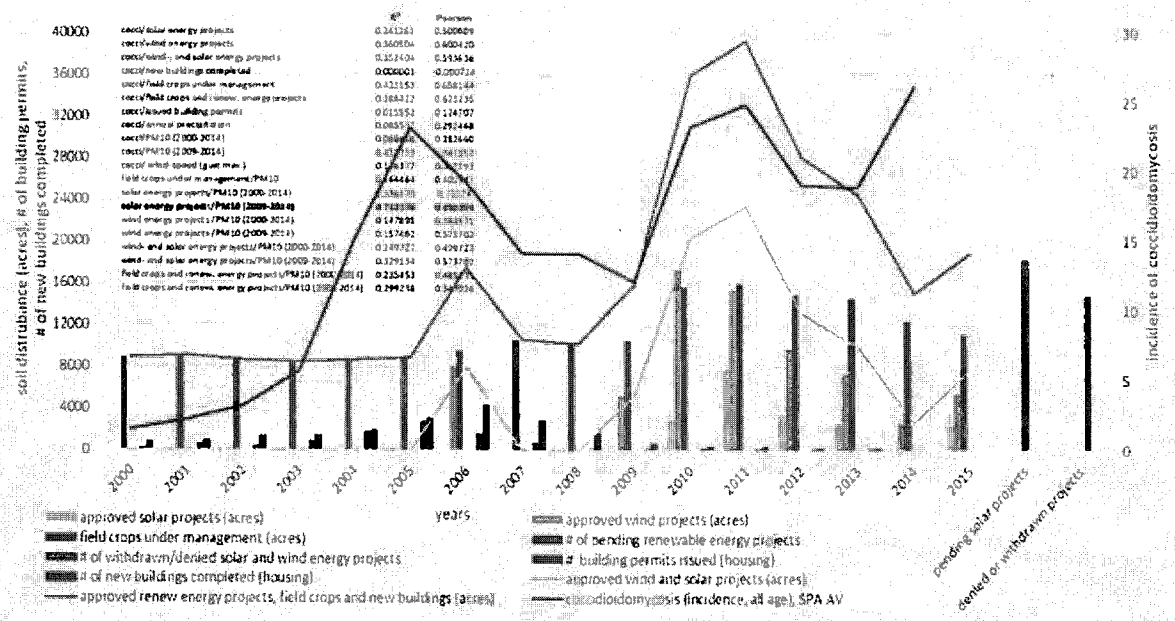


Fig. 6 Increase in soil disturbance over time in the Antelope Valley due to large-scale renewable energy project construction, changes in agricultural management correlated with incidence of coccidioidomycosis. Displayed are approved solar and wind projects (acres), agricultural fields under management (acres), as well as the number of issued building permits and completed buildings (housing developments) between 2000 and 2014. Included in the calculations (but not included in the graph) is also PM10 data (High National 24-Hour Average), precipitation (inches), and wind-speed data (gust max.) for the city of Lancaster measured at Foxfield Airport (KWJF). Solar and wind farms were graphed one year after the permit approval date because the begin of construction generally began in the year after permits were issued (data sources: www.arb.ca.gov/adam, http://publichealth.lacounty.gov/acd/Publications.htm, http://planning.lacounty.gov/assets/upl/project/energy_projects.pdf, http://pcd.kerndsa.com/planning/renewable-energy, http://lacfb.org/crop-reports-2/)

Table 4 Results of the initial multiple regression model: the model was coccidioidomycosis incidence = PM10 + precipitation +acres of land use (summed across Solar projects, wind projects, and active agricultural use

	Estimate	SE	t	p
(Intercept)	4.90	3.06	1.61	0.13
PM10	-6.14×10^{-3}	1.06×10^{-2}	−0.580	0.57
Annual Precipitation (in)	1.34	1.08	1.24	0.24
Acres of land use	4.04×10^{-4}	1.20×10^{-4}	3.36	0.0057**

Final model is: coccidiomycosis incidence = 4.9 − 0.0062 × PM10 + 1.33 precipitation + 0.00040 land use. $F_{3,12} = 4.8$, $p < 0.05$, multiple $r^2 = 0.54$

were indicative of the presence of the pathogen in the Southern San Joaquin Valley [44, 45]. The Southern San Joaquin Valley Desert is geologically somewhat related to the Western Mojave Desert where the Antelope Valley is located, but differs in elevation and climate [24]. Soils of both locations developed from quaternary alluvium and similar underlying parent material and have been described as alluvial fans or fan remnants and basin floors, with high concentrations of fine particulate matter that accumulated since the late Pleistocene and earlier. However, the pathogen was also detected in grassland from soils characterized as Greenfield sandy loam, Hesperia fine sandy loam, and Ramona fine sandy loam (sites 2, 3 and 5). The grassland appeared similar to a strong growth site of the pathogen, Sharktooth Hill near Oildale, east of

Table 5 Results of the 2nd multiple regression model: the model was coccidioidomycosis incidence = acres of solar projects + acres of wind projects + acres active agricultural use

	Estimate	SE	t	p
(Intercept)	−6.02	15.8	−0.38	0.711
Solar	8.86×10^{-05}	1.72×10^{-03}	0.052	0.96
Wind	5.16×10^{-05}	7.34×10^{-04}	0.070	0.945
Crop	1.78×10^{-03}	1.66×10^{-03}	1.07	0.306

$F_{3,12} = 2.3$, $p > 0.1$, multiple $r^2 = 0.36$

Bakersfield, not far away from a severely disturbed area, the Kern River oilfields, but the physical and chemical parameters of soils from Sharktooth Hill (high clay content) were more similar to those determined at site 6. Fossil diggers repeatedly became infected with *C. immitis* at Sharktooth Hill, where the presence of the pathogen has been confirmed repeatedly [20, 45, 64, 70]. Overall, soils from all *C. immitis* positive sites in the Antelope Valley and the Bakersfield area can be characterized as fine particulate sandy loam. The investigation of other soil types should be included in future studies to refine the set of environmental parameters that are indicative of the presence of the pathogen and to deepen our understanding of the ecology of *C. immitis* in California. The diversity of habitats that *C. immitis* can apparently grow in indicates that the pathogen is able to adapt to somewhat different soil environments or that different ecotypes of the pathogen exist which might explain its "spotty distribution" [6, 20]. Furthermore, it should be noted that site 6 where the pathogen was detected repeatedly had the lowest amounts of extracted DNA. A fungal species such as *Coccidioides* spp. which is missing some key enzymes needed to grow successfully as a saprophyte in soil might benefit from a low diversity of overall soil microbes that could include potential competitors and antagonists [65].

It has been difficult and expensive to detect *Coccidioides* spp. in soil and dust samples in the past [8, 22], but modern culture-independent molecular methods became available in recent years which allow for successful screening of environmental samples for the presence of *C. immitis* and *C. posadasii* [7, 36, 37, 42, 44–46, 66, 76]. However, soil analyses for the detection of soil-borne pathogens, such as *Coccidioides* spp., have not been included in Environmental Impact Reports (EIRs) for any construction project planned in the Antelope Valley or in other endemic areas of the pathogen in the Southwestern US. The scarcity of experts who are familiar with the procedures to detect the pathogen in its natural environment, additional costs of soil analyses, and a general underestimation of the risk of otherwise healthy people of contracting coccidioidomycosis from dust exposure might explain this potentially risky situation.

Mitigation and regulatory efforts are likely to be inadequate if the current trends in development and associated fugitive dust emissions continue. During spring 2014, fugitive dust emissions in the Antelope Valley negatively impacted air quality to an extent never documented before, reaching values up to and above 1000 μg/m^3, which reminded people of the Great Dust Bowl of the 1930's in Oklahoma [47], or the extreme dust storms documented in Owens Valley after the 110 mi^2 Owens Lake had been dried to support the water thirsty city of Los Angeles for a little more than a decade (1913–1926, feeding the Los Angeles aqueduct, see [60, 67]). Wilken et al. [78] indicated the inability of current dust mitigation strategies to protect construction workers from infections with *Coccidioides* spp. Lack of regulatory expertise and unrealistic expectations have resulted in costly failures of dust mitigation methods in the Western Mojave Desert in the past as described in McRae [52]. Environmental Impact Reports (EIRs) have been particularly criticized for not describing how dust mitigation measures are implemented and supervised, and no long-term dust control mitigation measures are included in the reports [73].

Mitigation and regulations are important considerations because some of the construction projects are in the immediate neighborhood of schools or close to human settlements. For example, the Del Sur Solar Project [Conditional Use Permit (Nos. 14-15 and 14-16)] is located adjacent to and upwind of Del Sur Elementary School. As of October 2012, the enrollment consisted of approximately 750 students in grades K-8 who would be directly and constantly affected by fugitive dust emissions because of daily westerly winds.

Although rarely implemented, potential mitigation procedures have been developed. Re-vegetation of disturbed land as a long-term strategy of dust control has been suggested by the Antelope Valley Dustbusters Taskforce, a group which consists of private

entities, as well as federal, city, and county government representatives [3, 29, 41], but the implementation of their recommendations into Dust Control Plans (DCPs) rarely occurred [10]. Based on 20 years of dust mitigation experience in the Antelope Valley, The Dustbusters Task Force of 1991 developed handbooks for farmer and homeowners in the Antelope Valley which are publicly available at (http://www.kernair.org/Main_Pages/information.html#; see also [18, 29, 61, 68]). Based on the findings of this study, we recommend that EIRs include soil analyses for *Coccidioides* spp. on land destined for construction of any type in endemic areas of the pathogen.

Conclusion

Although the change from non-renewable to renewable energy is generally welcomed in California, disturbing soils in endemic areas of a soil-borne pathogen that already causes disease incidence of epidemic character should only be considered if successful long-term dust mitigation measures are implemented, supervised, and controlled. The increasing demand for renewable energy shows promise for our planet in the future and will reduce some airborne emissions. However, there are hazards when sourcing new locations. One such danger is *Coccidioides* spp. arthroconidia becoming airborne when soil is disturbed and dust mitigation measures are inefficient or absent.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

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EXHIBIT B