

Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate

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ABSTRACT: The aetiological agent of amphibian chytridiomycosis *Batrachochytrium dendrobatidis* is a primary cause of amphibian population declines. Current surveillance is based on the detection of *B. dendrobatidis* in its host but *in vitro* work suggests infective stages may survive in the abiotic environment for at least 3 mo. We describe here a surveillance system using filtration and quantitative PCR that can detect *B. dendrobatidis* in small (<1 l) volumes of water. After assessing the analytical sensitivity of the protocol for both water and sediment samples in the laboratory, we analyzed environmental samples from the Sierra de Guadarrama mountain range in Spain at locations associated with chytrid-related die-offs and at other sites across Spain. *B. dendrobatidis* was detected in samples from 64 % of the ponds in the Sierra de Guadarrama and at 2 sites outside this region, showing that levels of amphibian exposure to *B. dendrobatidis* are spatially heterogeneous. In experimental microcosms, we detected *B. dendrobatidis* for up to 12 wk, though we found no evidence for an overall increase in biomass. Our results emphasise the need to further investigate the life cycle of *B. dendrobatidis* to more completely understand the epidemiology of this emerging pathogen.

KEY WORDS: Chytridiomycosis · *Batrachochytrium dendrobatidis* · Environmental surveillance · Filtration

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INTRODUCTION

The aetiological agent of amphibian chytridiomycosis *Batrachochytrium dendrobatidis* is highly pathogenic to some amphibians and is one of the leading causes of global amphibian declines (Berger et al. 1998, Longcore et al. 1999, Pounds, 2006). The species is the only member of the phylum Chytridiomycota (Kingdom Fungi) that parasitizes a vertebrate host (Berger et al. 1998). The phylum is ecologically diverse but all species have an unwalled, flagellated zoospore that requires an aqueous medium for its dispersal and survival. Many chytrids have a resistant resting stage (Powell, 1993), but no such structure has been identi-

fied for *B. dendrobatidis* (Morehouse et al. 2003). Zoospores produced by *B. dendrobatidis* infect the keratinized epidermis of amphibians and the mouthparts of anuran larvae, where they embed and form a zoosporangium. Detection of the zoosporangium in the host using either microscopic or molecular techniques has directed current surveillance strategies in amphibians (Berger et al. 1998, Garner et al. 2005).

Persistence of the organism in the environment in the absence of a host is not well understood. *Batrachochytrium dendrobatidis* can survive and remain infectious in sterile moist river sand for up to 12 wk (Johnson & Speare 2005). The organism can also grow on dead amphibian or snake skin and in liquid, or on

solid culture media (Longcore et al. 1999). Thus, zoospores may persist in the environment for long periods of time and there may be hitherto undetected saprophytic stages with the ability to multiply. To better understand the biology and to analyze the epidemiology of *B. dendrobatidis*, a technique that can detect the pathogen in the environment and outside the host is urgently required.

Traditionally, zoospore fungi are isolated with baiting methods using the bait a substrate for the fungus (Fuller 1987). This technique works well if the objective is to characterize an unknown fungal community, but is less effective if the objective is to isolate a particular species, especially if the target species is found at low densities or is poorly competitive. Previous attempts to obtain environmental samples of *Batrachochytrium dendrobatidis* by using traditional baiting techniques were not successful (Longcore et al. 1999, Livo, 2004).

The screening of DNA samples with environmental quantitative PCR provides a powerful tool to quantitatively detect water- and soil-borne pathogens, and its applications have been demonstrated for pathogenic *Candida* cells (Brinkman et al. 2003) and for *Perkinsus marinus*, a serious pathogen of the oyster *Crassostrea virginica* (Audemard et al. 2004). Here, we describe the development of a method that combines a simple, hand-held filtration system, a commercially available DNA extraction kit and a highly sensitive quantitative real-time PCR assay to detect *B. dendrobatidis* in small volumes (<1 l) of water. Our objectives were to (1) develop a sensitive, specific and quantitative tool to detect *B. dendrobatidis* in the environment, and (2) use this tool to assess spatial heterogeneity in levels of exposure of amphibians to *B. dendrobatidis*. The detection of *B. dendrobatidis* outside the amphibian host is vital for improvement of current surveillance measures and for more thorough study of fungus transmission at population and landscape levels.

MATERIALS AND METHODS

Minimum detection threshold for *Batrachochytrium dendrobatidis*. *B. dendrobatidis* was isolated from dead *Alytes obstetricans* samples collected in the Pyrenees mountains (isolate IA042, M. C. Fisher & J. Bosch, www.spatalepidemiology.net/bd/maps) and cultured using standard techniques (Longcore et al. 1999). Zoospores were harvested from cultures and counted using a haemocytometer. These were used to prepare 10-fold serial dilutions (1 ml of solution contained 1000, 100, 10, 1 or 0.1 zoospore equivalents) for subsequent spiking experiments. Stocks were stored at 4°C. Two

autoclaved turbidity matrices were prepared using water and sediment from Regent's Canal and the Serpentine, London, UK. Turbidities corresponded to the natural state of the source water (percentage packed volume [ppv] = 1.3%) and to approximately 3× the natural state (4% ppv). Turbidity values were measured after centrifugation (1000 × *g*, room temperature, 10 min) as the ppv of 10 ml taken from a sample (Lowery et al. 2000). One ml of each culture dilution was added to a conical centrifuge tube containing 50 ml of either 1.3% or 4% ppv solution.

Treatments (n = 3) and an untreated control were included in each matrix. The contents of each flask were filtered through a cellulose nitrate filter membrane (0.45 µm pore size to capture 1–2 µm zoospores; Berger et al. 1999) held in a dismountable 47 mm Swinnex filter holder (SX0004700, Millipore) using a 50 ml syringe (XX1105005, Millipore) to collect and concentrate any *Batrachochytrium dendrobatidis* present. Rather than assessing various eluting strategies, we disrupted whole filter membranes (Brinkman et al. 2003, Coupland & Johnson 2004). Following filtration, each membrane was placed into a Powerbead tube (Mobio Power Soil DNA extraction kit). These extraction kits were used due to their proven ability in removing environmental PCR inhibitors and for their high binding capacity of 20 µg of DNA that limits saturation effects. The manufacturer's protocol was adopted with modifications. Specifically, Step 5 of the protocol was modified by homogenizing samples with a Minibeadbeater 8 (Biospec Products) at maximum speed for 2 min, rather than using the recommended vortex for 10 min. At the final step, 100 µl of supernatant were collected, diluted 1:10 and stored at –20°C. The DNA dilutions were screened following the real-time quantitative Taqman assay of Boyle et al. (2004) in an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS). High analytical sensitivity and specificity were achieved using primers ITS-1 Chyt and 5.8S Chytr, which target this multicopy region of the genome. A minor groove binder probe (ChytrMGB2) was used for detection. Samples were prepared in duplicate. Amplification standards of 0.1, 1, 10 and 100 zoospore equivalents and a negative control (distilled water) were included. The amplification standards were prepared from an isolate of known cell density (IA042 *B. dendrobatidis*, haemocytometer count). In the ABI 7300 SDS, amplification standards produced a standard curve from which quantities of *B. dendrobatidis* (as number of zoospore equivalents, i.e. ZE) in unknown samples were calculated (Fig. 1). A sample was identified as positive if clear log-linear amplification with a change in fluorescence ($\Delta R_n > 0.1$) was observed in both duplicates. Quantities were

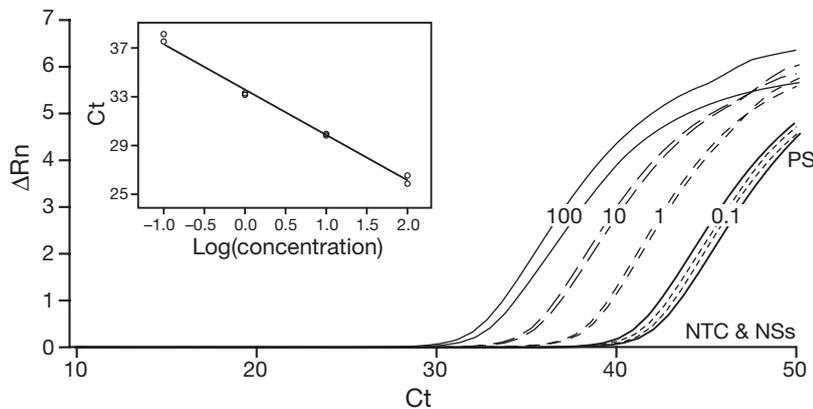


Fig. 1. *Batrachochytrium dendrobatidis*. Amplification plots of DNA extractions outside the core study site in the Sierra de Guadarrama. Change in fluorescence (ΔRn) is plotted against cycle threshold number (Ct). Standards (0.1, 1, 10, 100 zoospore equivalents), the negative control (NTC), and the extractions corresponding to a positive sample (PS) and a negative sample (NS) are labelled. The multiple curves indicate duplicates for each sample. Inset: quantification standard curve. $Ct = -3.7 \times \log(ZE) + 34$, where ZE represents number of zoospore equivalents in the well

estimated according to a sample's cycle threshold (Ct) value, i.e. the cycle number at which $\Delta Rn = 0.1$.

Disinfection procedure for filter housing and syringe. Sodium hypochlorite is an effective agent for killing *Batrachochytrium dendrobatidis* (Johnson et al. 2003) and for denaturing nucleic acids (Suarez et al. 2003). The effectiveness of a disinfection procedure using 5% sodium hypochlorite (domestic bleach) was assessed in the laboratory prior to its use in the field. After flushing 75 ml of a solution containing 1.5×10^6 zoospores through the filtration system, the filter housing and syringe were immersed in a 5% sodium hypochlorite solution for 5 min. The components were then washed and flushed with tap water to remove any residues of sodium hypochlorite. On reassembling the filtration system, 75 ml of distilled water were passed through a new membrane. The membrane was removed and the presence of *B. dendrobatidis* DNA was checked using the extraction and detection procedure described above.

Microcosm experiments. In 2005 and 2006, microcosms containing autoclaved and non-autoclaved pond sediment were established. In 2005, eight 5 g samples of sediment were inserted into 50 ml centrifuge tubes. Half of the tubes were autoclaved thrice at 121°C for 60 min, with intervals of 24 h at room temperature between sterilizations. Subsequently, 3 of the 4 tubes from each treatment (autoclaved/non-autoclaved) were spiked with 500 μ l of culture containing the equivalent of 1.5×10^6 zoospores ml^{-1} . The remaining 2 tubes served as negative controls and were spiked with 500 μ l of uninoculated culture medium. The microcosms were incubated in a non-rotating,

artificially lit, vented incubator at 18°C for 9 wk. After 0, 3, 7 and 9 wk of incubation, the tubes were vortexed briefly and 3 samples of 0.25 g (wet weight) of sediment were collected. DNA was extracted from the sediment and amplified by quantitative PCR (qPCR) as described above. This experimental set-up was repeated in 2006 with the following amendments: (1) each treatment was replicated in triplicate, and (2) samples of sediment were collected at Weeks 0, 4, 8 and 12.

Assessment of the technique in the field. The field study was conducted primarily in and around Peñalara Natural Park (768 ha, 40° 50' N, 3° 57' W), an alpine region located between 1800 and 2200 m elevation in the Sierra de Guadarrama mountain range of central Spain (Fig. 2A). Mass mortality of *Alytes obstetricans* attributable to chytridiomycosis

was first detected here in 1997 (Bosch et al. 2001) and all 9 amphibian species in the park have been infected by *B. dendrobatidis* (S. F. Walker, M. C. Fisher & J. Bosch unpubl. data). Peñalara contains over 250 bodies of water; some are ephemeral and others permanent, ranging in size from 0.3 to 6463 m^2 . The waters are slightly acidic (pH 6) with low levels of conductivity ($<30 \mu S cm^{-1}$ at 25°C) (Toro & Granados 1999).

In July 2005, we sampled within the Sierra de Guadarrama at 42 sites for water and 52 sites for sediment. Water was collected within 10 cm of the edge of a pond and passed through the filter until the membrane was saturated. When streams flowed from pools, filtrations were conducted at the points of confluence to maximize representation of complete water bodies. Each membrane was folded twice with sterile forceps, with the 'capturing surface' facing inwards. The membranes were stored individually in 60 ml sterile Nasco Whirlpak™ polyethylene bags, and kept on ice until frozen at -20°C. DNA was extracted and screened from these membranes. The relative density of *Batrachochytrium dendrobatidis* at each site was calculated from the estimated number of zoospores (as determined by quantitative PCR) and the volume of water filtered. To account for the 10-fold dilution factor and the use of only 5 μ l per 100 μ l total extraction volume as a PCR template, the extracted zoospore equivalent (EZE) was calculated as: $EZE = ZE \times 10 \times 20$. $EZE l^{-1}$ was calculated as: $EZE l^{-1} = (EZE/filtrate volume (ml)) \times 1000$. To reduce cross-contamination, the collecting and filtration system was cleaned in the field by immersion in a solution of 5% sodium hypochlorite for

at least 5 min before and after each filtration. Additionally, prior to the start of a new filtration, the system was flushed 10 times with water from the new sample site.

Superficial sediment was collected from each water filtration site and from 8 additional ponds in which no water remained. Approximately 100 g of sediment was collected in conical centrifuge tubes and excess water was eliminated by decanting. Samples were stored in sterile Whirlpak polyethylene bags and kept on ice until frozen at -20°C . From each sample, 0.25 mg (wet weight) was processed using a Mobio Power soil DNA extraction kit.

Additional filtrations were conducted from 29 other sites across Spain where amphibian populations have been tested for the presence of *Batrachochytrium dendrobatidis* (Fig. 2). These sites represent a diverse array of water bodies (lakes, streams, water troughs) across a broad altitudinal range.

Assessment of environmental PCR inhibitors. An internal positive control was used to assess evidence for PCR inhibition. Following the methodology of Hyatt et al. (2007), a VICTM labelled synthetic amplicon was used as an internal positive control (VICTM dye, Applied Biosystems No. 4308323). For a subset of samples, this internal positive control was included in one of each duplicate well as 1 μl 10 \times Exo IPC mix and 0.5 μl 50 \times Exo IPC DNA (Applied Biosystems). Environmental samples with Ct values significantly higher than a control were considered to be under the influence of PCR inhibition.

Statistical methods. Statistical analyses were conducted with the R software package (Version 2.2.1, The R Foundation for Statistical Computing). The Wilcoxon-Mann-Whitney test was used to test for significant differences in the recovery of DNA between the autoclaved and non-autoclaved microcosms at each time period. Significance was assessed at the 5% level. In 2006, mean Ct values from the 3 samples collected in each tube were used in the analysis.

RESULTS

Optimization of the technique and disinfection procedure

The minimum detectable spike of one zoospore (0.9 genome equivalents) in 50 ml of water was detected in both water matrices. At each concentration, the amount of DNA detected (as $1/\text{Ct}$) was lower in the more-turbid water matrix (paired t -test, $p < 0.005$). No positive amplifications were obtained from membranes used in the apparatus post-disinfection showing that there was no carry-over of zoospores or of detectable *Batrachochytrium dendrobatidis* DNA.

Persistence of *Batrachochytrium dendrobatidis* DNA in experimental microcosms

In both years, *Batrachochytrium dendrobatidis* was detected for the entire duration of the experiment, except in the negative control microcosms. However, between the first and the last sampling period, recovery declined by up to and $>99\%$ (Fig. 3). At all of the

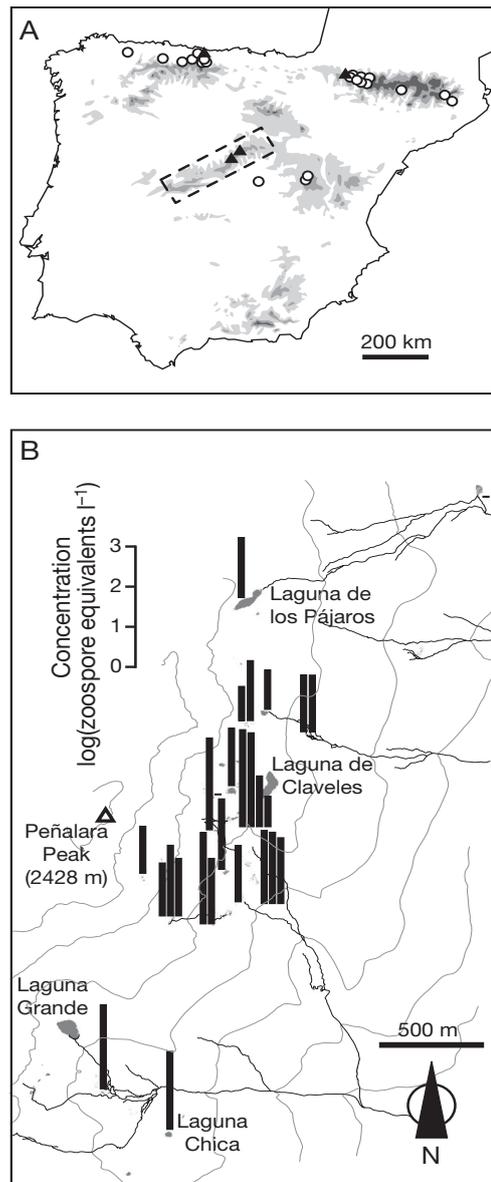


Fig. 2. *Batrachochytrium dendrobatidis*. (A) Locations of sampling sites in Spain. Sites positive (▲) or negative (○) for the fungus. Peñalara Natural Park is located within the Sierra de Guadarrama mountain range (box). (B) Sites within Peñalara Natural Park that tested positively for *B. dendrobatidis*. Bar charts show concentrations of zoospores at sites (as log zoospore equivalents l^{-1}). Zoospore concentrations in samples of known filtrate volumes were calculated from amounts of DNA recovered from filters

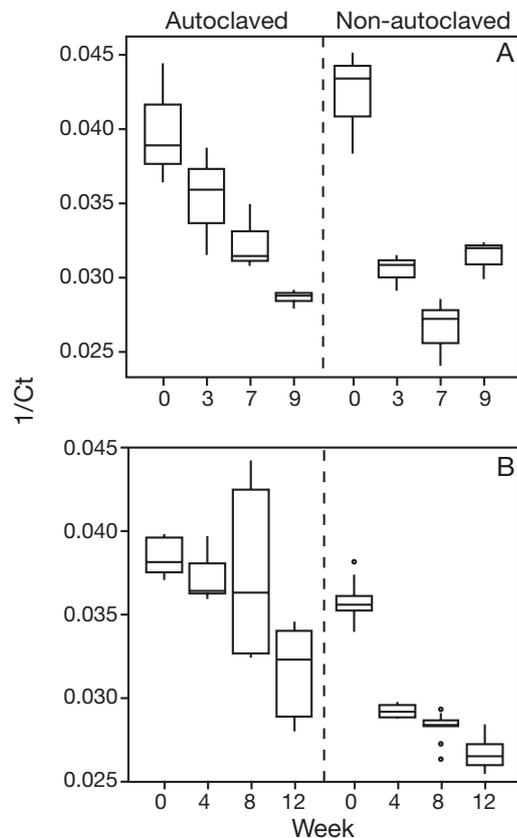


Fig. 3. *Batrachochytrium dendrobatidis*. Decrease in target DNA (increase in 1/cycle threshold, 1/Ct) plotted against week post-inoculation in (A) 2005 (n = 3) and (B) 2006 (n = 9); in both plots, numbers on the x-axis refer to weeks post-inoculation. Treatment is autoclaving. Box and whisker plots show medians, interquartile ranges and min./max. values

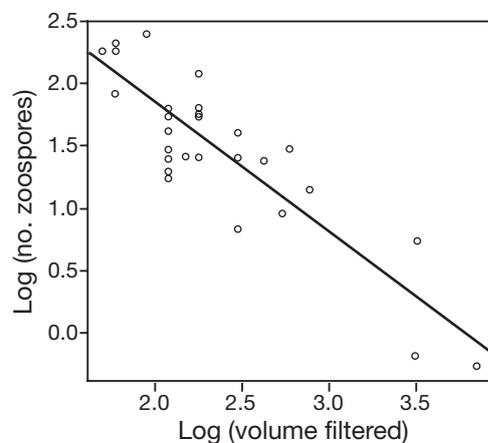


Fig. 4. *Batrachochytrium dendrobatidis*. Negative relationship between the volume (ml) of filtered water and the concentration (no. l^{-1}) of zoospores recovered on the filter; both variables log transformed. Surveillance sites are located within or in close proximity to Peñalara Natural Park (Sierra de Guadarrama) (see Fig. 2)

time periods in 2006 and at 2 of the 4 sampling periods in 2005, the Ct values in the autoclaved microcosms were significantly lower (i.e. greater mass of *B. dendrobatidis* DNA) than those in the non-autoclaved microcosms (Wilcoxon-Mann-Whitney test, $p < 0.05$).

Field detection of *Batrachochytrium dendrobatidis* in water and sediment

With the exception of 4 water bodies, <1 l of water was filtered at each site, and <200 ml were filtered at >50% of the sites, reflecting the highly turbid nature of the waters. We detected *Batrachochytrium dendrobatidis* on 64% of the filters from ponds in the Sierra de Guadarrama. The estimated concentration of *B. dendrobatidis* at the positive sites varied from 0.5 to 262 zoospores l^{-1} (Fig. 2B), with a median of 31 l^{-1} , an interquartile range of 20 to 67 l^{-1} and a SD of 70.5 zoospores. At Circo del Nevero, where there was an outbreak of chytridiomycosis at the time of sampling, we detected 67 zoospores l^{-1} . A highly significant (linear regression adjusted $r^2 = 0.75$, $F_{1,26} = 82.02$, $p < 1 \times 10^{-8}$) negative relationship was found between the volume of water filtered (which is inversely related to turbidity) and the calculated concentration of *B. dendrobatidis* zoospores (Fig. 4), i.e. the more turbid the water, the higher the concentration of *B. dendrobatidis*.

Batrachochytrium dendrobatidis PCR products were not obtained from 48 of the 52 DNA extractions from sediment. For the remaining 4 samples, amplification occurred in only one of the replicate wells; we consider these 4 results to be indeterminate.

Samples from outside the Sierra de Guadarrama mountain range

Batrachochytrium dendrobatidis was detected in 2 of the 29 samples taken outside the Sierra de Guadarrama mountain range (Fig. 2A). One site was a water trough in Asturias where larvae of *Alytes obstetricans* have been found PCR-positive for *B. dendrobatidis* (S. F. Walker & J. Bosch unpubl. data). The second site was a lake in the Pyrenees, Ibon Acherito, where mass mortalities of *Alytes obstetricans* have been observed (M. C. Fisher & J. Bosch unpubl. data).

Assessment of environmental PCR inhibitors

Ct values corresponding to the amplification of the synthetic amplicon in the internal positive control showed no evidence of PCR inhibition in either the

more or the less turbid artificial water matrices. In Peñalara, 3 of the 6 sediment samples screened with the internal positive control showed no evidence of PCR inhibition (i.e. Ct values were within 0.5 of the negative extraction control). One sediment sample was within 2 Cts of the negative extraction and 2 samples showed significant evidence of inhibition (in one of the samples the amplicon did not amplify and in the second there was a delay of 19 cycles). None of the 16 filter extractions from pond water screened with the IPC showed any evidence of PCR inhibition.

DISCUSSION

This study focused on developing a protocol for detecting *Batrachochytrium dendrobatidis* beyond the host. Monitoring of environmental stages of the fungus (i.e. outside the host) is essential for an understanding of its epidemiology because pathogens that cause high host-mortality are subject to density-dependent regulation and rarely cause extinction. This is because transmission chains in highly virulent pathogens with single host species tend to end when the basic reproduction number R_0 (average number of secondary cases of disease produced per typical primary case [Anderson & May 1991]) is <1 . However, if a pathogen has multiple hosts, or a reservoir of stages outside any biological host, values of $R_0 > 1$ can be maintained throughout the decline of a susceptible host population or species. Under these conditions, high levels of infection leading to extinction of susceptible hosts can occur if (1) the host species are highly endemic, i.e. spatially constrained, and (2) both within- and between-species transmission rates are high (Fenton & Pedersen 2005). The high mortality rates suffered by amphibian species suggest that *B. dendrobatidis* may have a reservoir of stages outside the hosts (Daszak et al. 1999). Accordingly, we focused on methods for detecting fungi in the environment.

We found that *Batrachochytrium dendrobatidis* can be detected in the natural environment outside its amphibian host at densities of up to 262 zoospore equivalents l^{-1} . However, we stress that our test does not distinguish DNA from cells, or actively reproducing cells from non-reproductive infective ones. The prevalence of *B. dendrobatidis* in certain areas of Spain (e.g. Peñalara Natural Park) is consistent with high values for R_0 and with the existence of large numbers of infectious zoospores. We hypothesize that the natural concentration of infectious zoospores predisposes susceptible amphibian populations to extirpation. We demonstrated high concentrations of *B. dendrobatidis* in regions undergoing mass mortalities. Therefore, there is an urgent need for further ecologi-

cal studies of *B. dendrobatidis* to provide input data for current epidemiological models that consider transmission dynamics in multi- and single-host communities (Briggs et al. 2005).

The qPCR assay we employed is analytically sensitive to 0.1 zoospore equivalents and is highly specific for *Batrachochytrium dendrobatidis* (Boyle et al. 2004, Hyatt et al. 2007). The analytical specificity of the assay has been confirmed against 26 other Chytridiomycetes from 4 orders, viz. Chytridiales (*Rhizophydium* sp., *Rhizophlyctis* sp., *Cladochytrium* sp. and *Chytrium* sp.), Monoblepharidales (*Gonapodya* sp.), Blastocladales (*Allomyces macrogynus*, *Catenaria* sp., *Catenophlyctis* sp.) and Spizellomycetales (*Spizellomyces* sp., *Rhizophydium* sp., *Powellomyces* sp.; Boyle et al. 2004, Hyatt et al. 2007). Further, our finding that positive environmental qPCR results correlate with known areas of infection is consistent with high diagnostic specificity.

Beyond Peñalara and the Sierra de Guadarrama mountain range, 2 of 29 water bodies tested were positive for *Batrachochytrium dendrobatidis*. One of these was a water trough in Asturias where larvae of *Alytes obstetricans* infected by *B. dendrobatidis* were present at the time of sampling (S. F. Walker & J. Bosch unpubl. data) and the other corresponded to a lake in the Pyrenees from which chytrid-related mass mortalities of *A. obstetricans* have been detected. Because we screened amphibians at all the filtration sites (S. F. Walker & J. Bosch unpubl. data), we know that *B. dendrobatidis* is present at some sites that tested negative for the fungus in our molecular screening of filtered water. At these sites, the infection load in the environment may be lower than the detection threshold of our test, and/or micro-scale spatial and temporal heterogeneity may be preventing detection.

The significant association between the concentration of *Batrachochytrium dendrobatidis* and the relative turbidity of the waters in the Sierra de Guadarrama (Fig. 4) suggests that more complex water matrices may favor the survival of this fungus. If turbidity is a proxy for biological activity, this relationship is counterintuitive to our finding that the density of *B. dendrobatidis* was almost always consistently higher in the autoclaved than in the non-autoclaved microcosms. Thus, as in other aquatic microbial communities, species composition and competitive interactions may have a stronger regulatory effect on the growth dynamics of *B. dendrobatidis* than richness per se. The regulatory potential of the biotic environment will itself be modified by the abiotic environment, 2 effects that are difficult to disentangle in the complex matrix of water bodies in Peñalara. Notably, the absence of amplifiable *B. dendrobatidis*-DNA in the superficial sediment of ponds suggests that physico-chemical con-

ditions limit the survival of *B. dendrobatidis*. Given that 3 of 6 sediment samples screened using the IPC showed no evidence of PCR inhibition, it seems unlikely that inhibitors in the sediment can completely explain the absence of *B. dendrobatidis*.

Previous work has suggested that *Batrachochytrium dendrobatidis* may have a naturally occurring saprophytic stage. The species may survive and remain infectious in sterile, autoclaved lake water for up to 7 wk post-inoculation (Johnson et al. 2003) and in sterile moist river sand (pH 5.8) for at least 12 wk (Johnson & Speare 2005). In addition to growth in or on culture media (Longcore et al. 1999), the organism also can grow on dead amphibian and snake skin, dead algae, insect exoskeletons and feathers (Johnson & Speare 2005). Despite its sensitivity to desiccation, *B. dendrobatidis* also can survive on feathers that have been dried for 1 to 3 h (Johnson & Speare 2005).

Although *Batrachochytrium dendrobatidis* is keratinophilic (Daszak et al. 1999), the fact that not all chytrid species are dependent on a single substrate for either attachment or metabolism (Sparrow 1960), together with the aforementioned laboratory and field studies, suggests that *B. dendrobatidis* may have a more complex epidemiological profile than would be expected for a pathogen dependent upon only amphibian hosts. The detection of *B. dendrobatidis* in small volumes of filtered water certainly supports this hypothesis. However, there was no consistent increase in biomass in the microcosms, demonstrating clear limits to the ecological envelope within which survival and growth are possible.

Numerous other ecological factors may affect abundance of *Batrachochytrium dendrobatidis* in the environment. The relatively low turnover rate in the waters of Peñalara may allow an increase in the concentration of cells, facilitating detection (by molecular technology) and the formation of significant abiotic reservoirs of zoospores outside the host animals. Further, the concentrations of *B. dendrobatidis* that we recorded in 2005 may have been particularly high as a result of abnormally low water levels within the park; 2005 was the driest year recorded since record keeping began in 1947 (see: www.inm.es). The levels of *B. dendrobatidis* in riparian habitats are expected to be lower than in the ponds of Peñalara due to continuous flushing of the system. Hence, environmental surveillance of *B. dendrobatidis* may demand a system that can process larger water volumes than we have described here.

The actual relationship between the DNA level detected and the number of viable cells present remains unknown. Extracellular DNA bound to sediment, for example, may be physically protected from nucleases. In contrast to DNA and rRNA, mRNA is turned over rapidly within cells, typically with a half

life of only a few minutes, and its presence has been correlated with cell viability (Sheridan et al. 1998). Hence, the use of mRNA in reverse transcriptase PCRs enables both the symptomatic and presymptomatic detection of viable fungal pathogens in plants (Gough & Lee 1985, McMaugh & Lyon 2003) and we aim to investigate cell viability using *Batrachochytrium dendrobatidis*-specific probes to the mRNA of a constitutively expressed gene such as beta tubulin (Yan & Liou 2006) or RNA polymerase RPB2.

Over 8 yr have passed since *Batrachochytrium dendrobatidis* was first described (Berger et al. 1998, Longcore et al. 1999). Although the implications of saprophytic nutrition in this species were highlighted in initial publications (Daszak et al. 1999, Speare 2001), the prevailing view today is that *B. dendrobatidis* is an amphibian-specific pathogen. In the natural environment, the survival and reproduction of portions of the organism's life cycle that are unrelated to the amphibian host remain unexplored. If, as is the case with many other chytrid species, *B. dendrobatidis* has a more complex biology than is currently appreciated (and hence a more complex epidemiological profile) the transmission parameters used in current theoretical mathematical models will not accurately reflect population dynamics and might even misguide conservation efforts. Quantitative environmental diagnostic tools such as those described here are essential for undertaking spatial and temporal surveys required for gaining a more complete understanding of the environmental reservoirs of *B. dendrobatidis* and the ecological constraints determining survival.

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