

Do salinity and pH help protect natterjack toads from chytridiomycosis, a disease caused by the amphibian fungus *Batrachochytrium dendrobatidis* (B.d.) ?



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LIST OF ACRONYMS

ARG	Amphibian and Reptile Groups
BSA	Bovine Serum Albumin
Ct	Count Types (Threshold Cycle)
EPH	Endemic Pathogen Hypothesis
GE	Genome equivalents
GLM	Generalised Linear Models
GPS	Global Positioning System
IoZ	Institute of Zoology, Zoological Society of London
IPC	Internal Positive Controls
RACE	Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity
NaCl	Sodium Chloride
NPH	Novel Pathogen Hypothesis
PCR	Polymerase chain reaction
rt-PCR	Real Time Polymerase chain reaction
UV-B	Ultraviolet-B
ZSL	Zoological Society of London

ABSTRACT

Chytridiomycosis is an amphibian disease caused by a fungal pathogen, *Batrachochytrium dendrobatidis* (*B.d.*), which has been instrumental in the global decline of amphibian populations. *B.d.* has recently been detected in the UK, including within six natterjack toad (*Epidalea calamita*) populations in Cumbria.

Previous research in the vicinity of coastal Cumbria in 2009-2011 has identified an apparent pattern between the infection levels of *B.d.* and salinity. Natterjacks, captured from brackish ponds exhibit *B.d.* infections that have been markedly weaker, in comparison with those from freshwater environments. Studies elsewhere have found evidence that the growth and infectivity of pathogens are strongly affected by environmental co-factors.

The primary objective of this study was to measure the effect of two environmental factors on the prevalence and presence of *B.d.* at coastal and inland natterjack sites across North West and Southern England. This study also measured the effects of these environmental factors on the performance of a *B.d.* detection method.

Results showed that both environmental factors had a significant association with the prevalence and presence of *B.d.* at natterjack sites. The sensitivity of the *B.d.* detection method was influenced by the presence of contaminants within the samples. Changes were implemented through the use of reagents, and the effect of contamination was reduced.

In conclusion, this project has highlighted the importance of two environmental factors on the prevalence and presence of *B.d.*; however this has been concomitant with a reduction in sensitivity of detecting *B.d.* in contaminated material. Further research is required to truly identify the long-term effects environmental factors are having on *B.d.* presence and prevalence.

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1. Introduction

Chytridiomycosis (*Batrachochytrium dendrobatidis*, *B.d.*) is an emerging fungal disease which has been instrumental in the decline and extinction of amphibian populations worldwide. Its virulent nature and ability to infect different species and individuals so variably has concerned and attracted the attention of epidemiologists and ecologists worldwide (Briggs *et al*, 2010). Its low host specificity likely contributes to the high probability of infection and establishment of *B.d.* if introduced locally. This is to some degree reflected by its broad host and geographic distribution. These attributes as well as its ability to survive at low host densities have enabled *B.d.* to have a profound impact on entire communities and ecosystems (Fisher *et al*, 2009).

Chytrids are important components of freshwater ecosystems; however measuring the nature and effects of interactions between parasites and their hosts is often difficult to quantify due to the unpredictable nature of population dynamics (Lafferty & Gerber, 2002).

Chytrids are particularly susceptible to a number of different physical and biological factors, which effect their growth and impact on their population size and composition (Gleason *et al*, 2008). *B.d.* in particular is influenced by the presence of wide-ranging environmental covarieties within different environments, which modify the host-*B.d.* interaction and thus affect the prevalence and presence of the pathogen (Santaliestra *et al*, 2011). Studies suggest that *B.d.* is especially vulnerable to desiccation, high and low temperature, low pH, UV-B radiation, as well as many fungicides and bactericides (Johnson *et al*, 2003; Santaliestra *et al*, 2011). Sodium Chloride (NaCl) has also been highlighted as a potential inhibitor (White, 2006). Studies have also emphasised the importance of synergistic interactions as multiple stressors on the host's ecological responses (Santaliestra *et al*, 2011).

Salinity and pH have been highlighted as potential important environmental site factors through recent research in Cumbria. Results have indicated that *B.d.* infection levels appear to be weaker in natterjack toads collected from brackish ponds, in comparison with those gathered from freshwater (Minting, pers. comm.). As a result, it's thought that salinity and pH may have protective effects, by killing or inhibiting *B.d.* at these sites. If this is the case, there could be substantial implications for management techniques at natterjack sites.

Research by White (2006), made a convincing argument that at concentrations above 0.5% Sodium Chloride (NaCl) chytrid zoospores were inactivated, however limited field research has investigated the possibility of this inhibitor as a defence against chytridiomycosis, and a future management tool.

1.1 Problem being addressed

Amphibians have declined dramatically over the last twenty-five years in many areas. Although many declines are attributed to habitat loss and over-exploitation, other, unidentified processes threaten 48% of declining species (Stuart *et al*, 2004). Declines are taxonomically random (Stuart *et al*, 2004; Bielby *et al*, 2008), and often at small geographical scales (Bielby *et al*, 2006), indicating that individual species attributes may influence susceptibility to decline.

Chytridiomycosis has been recognised as one of the causative agents of these amphibian declines, and has recently been detected in *E.calamita* populations in the UK.

The first published evidence of chytridiomycosis in Britain was in introduced Northern American Bullfrogs on the Sussex/Kent Border (Cunningham *et al*, 2005). Molecular diagnostics on DNA extractions of toe clip samples confirmed the presence of chytridiomycosis. Subsequently, during 2006/2007 infection was detected in four native amphibian species at six natterjack (*Epidalea calamita*) toad sites in Cumbria. Cumbria is recognised as an important stronghold for natterjacks, as it currently supports 50% of all UK natterjack sites. Habitats also consist of a mixture of heath, open mosaic and coastal habitats which are ideal for breeding.

Due to the decimating declines this pathogen has been linked to, it is crucial to investigate the extent of *B.d.* distribution on a nationwide basis. Also whilst monitoring of amphibian populations will become increasingly important, if global declines are to be corrected, preventative and control measures need to be developed and implemented (Buckley & Beebee, 2004). Therefore researching the influence environmental factors have on the presence, prevalence and severity of infection will be of particular importance.

The project focus was to investigate the observed pattern of *B.d.* infection in relation to site salinity and pH, which has been detected in an existing study, and to develop a statistically defensible position on the relationship between salinity and pH, and the presence and prevalence of *B.d.*

If the hypothesised trends are found to have a significant association with *B.d.* prevalence this could have a considerable effect on the future management of natterjack populations. Future location strategies and pond maintenance work could be harmonised with tidal zones to ensure ponds receive a regular inundation from saline water, maintaining the pond at an optimum level for natterjack toads and limiting the prevalence of *B.d.* respectively.

1.2 Aims and objectives

The aims of this study are;

- 1) To examine the influence of two environmental factors on the presence, probability of infection and severity of infection of *B.d.*

- 2) To identify the *B.d.* distribution across coastal *E.calamita* amphibian sites in the North West of England.
- 3) To measure and correct for, the effect of environmental factors on a *B.d.* detection method.

The objectives of this study are;

- 1) To investigate the pattern of salinity and pH as site factors that could affect the prevalence of *B.d.*
- 2) To investigate the hypothesis that *B.d.* scores correlate with pond salinity, by swabbing toads found within freshwater and saline ponds.
- 3) To investigate the hypothesis that *B.d.* scores correlate with pond water pH, by swabbing toads and recording pond pH.
- 4) To test for the presence or absence of synergistic effects between salinity and pH.
- 5) To investigate the prevalence and infection status of natterjack toads at *E.calamita* sites in North West and Southern England
- 6) To identify regional patterns in *B.d.* distribution, by conducting spatial comparisons amongst Cumbrian natterjack populations.
- 7) To test for the presence and absence of environmental effects on the *B.d.* detection method using Internal Positive Controls (IPC).

All of the above objectives will be completed within and across sites where possible, to maximise the opportunity for statistical comparisons.

The hypotheses to be tested in this study are;

- Pond salinity has a statistically significant correlation with the presence, prevalence and strength of *B.d.* infection in wild natterjack toads (independent of other site specific factors).
- pH has a statistically significant correlation with the presence, prevalence and strength of *B.d.* infection in wild natterjack toads (independent of other site specific factors).

1.3 Thesis Structure

The background information in section two provides a critical analysis of the previous work conducted. Firstly it focuses on an introduction to *B.d.* and the life cycle dynamics of this virulent fungus. Then the host-parasite dynamics and ecological factors and mechanisms that influence these dynamics are discussed. Finally literature highlighting the inhibitory effects that can affect rt-PCR as a method for DNA detection is analysed.

Section three outlines the details of the methodological framework, and describes the fieldwork, laboratory and statistical techniques used.

Section four reports the results of applying those methods and statistics.

Section five discusses the results of the analysis in a broader context, examines the limitations of the project, and discusses recommendations for the future in terms of research and management.

2. Background

2.1 Amphibian Declines

The loss of global biodiversity is a major international concern, with growing unease that amphibian populations are declining globally. However much of the supporting evidence and research is anecdotal or from studies that have been undertaken at small geographical scales and over short time periods (Lips *et al* 2006; Houlahan *et al*, 2000).

This presents complexities with establishing temporal trends in highly dispersed and variable populations, whilst also raising concerns about the validity of extrapolating global trends from local or regional studies. In an attempt to provide quantitative evidence for global amphibian population declines Houlahan *et al* (2000) examined data from 936 populations to assess large-scale temporal and spatial variations. They observed rapid declines from the late 1950s/early 1960s, followed by a reduced rate of decline to the present. Temporal variation was apparent in Western Europe and North America where population trends were negative during the 1960s, but only the latter showed declines from the 1970s to the 1990s. This indicates that there is considerable geographical and temporal variability amongst populations. This also signifies that amphibians have declined globally over the past decade and continue to do so (Houlahan *et al*, 2000).

The decline of amphibians in Britain has traditionally been attributed to habitat change through afforestation, urbanisation, pollution through unsustainable agricultural practices, seral succession on neglected heathlands, acidification of breeding ponds and invasion by competitively superior species (McGrath & Lorenzen, 2010). For instance, the natterjack toad has suffered devastating declines due to loss of habitat, through the conversion of prime early successional habitat, and due to anthropogenic acidification of vital breeding sites (Denton *et al*, 1997).

In addition to these threats, there have been cases of rapid disappearance in areas where no direct human disturbances have been recorded (Wake, 1991). Factors that have been highlighted as the cause of these declines include; emerging infectious diseases, increased UV-B radiation, high acidification and climate change. Furthermore a particularly striking impact is the ability of pathogens to affect amphibian populations so variably. These host-parasite interactions have been known to cause effects at different life stages, resulting in developmental and physiological deformities, or in severe cases proximate mortality (Blaustein & Kiesecker, 2002).

However despite the limited recognition of these problems, research programmes concentrated on understanding species losses typically focus on the direct effects of single factors. Yet recent studies suggest that global amphibian losses are the result of interactions between numbers of highly context-dependent causal factors (Blaustein & Kiesecker, 2002; Becker & Zamudio, 2011). Therefore multi-factor studies investigating stressors that may

compromise disease defence mechanisms are warranted if we are to understand the complex nature of host-parasite dynamics (Kiesecker & Blaustein, 1995).

2.2 Chytridiomycosis

One of the many threats facing amphibian species and population survival worldwide is the disease chytridiomycosis, caused by the chytrid fungus, *Batrachochytrium dendrobatidis* (*B.d.*) (Weldon *et al*, 2004). It has been implicated in epizootic mass mortality globally, has a wide host range and is linked to species declines, and local population extinctions (Lips *et al*, 2006; Bosch *et al*, 2001; Pounds & Crump, 1994). In the montane and tropical regions of Central America, mass die-offs have led to rapid population declines. For example Lips *et al* (2006) observed a link between the appearance of *B.d.* and mass mortality in an amphibian community in Panama. Additionally, Pounds & Crump (1994) suggest that chytridiomycosis may have been the proximal cause of a climate-change driven extinction of the golden toad (*Bufo periglenes*), and mass declines of the harlequin frog (*Atelopus varius*) in Costa Rica. In the United Kingdom the presence of *B.d.* in natterjack toads has already been recorded at multiple sites throughout the North West of England (Arai, 2008). Considering the widespread and rapid declines which have occurred in other regions, this is particularly worrying for the future of the natterjack toad and other native amphibian populations.

2.2.1 Life Cycle of *Batrachochytrium dendrobatidis*

The life cycle of *Batrachochytrium dendrobatidis* (*B.d.*) has two life stages; [1] Sessile, reproductive zoosporangium that produces zoospores asexually and [2] motile, infectious zoospores (Berger *et al*, 2005).

Optimal growth occurs between 17 -25°C and a pH of 6-7 *in-vitro*, but trade-offs exist between the zoosporangium maturation rate, and the number of zoospores produced, such that similar population growth rates can be achieved across a range of temperatures (Woodhams *et al*, 2008). Nonetheless, several strains show reduced growth, and/or mortality above 28°C, and this appears to influence infection patterns (Piotrowski *et al*, 2004). Temperatures can change dramatically across seasons, altitudes and latitudes, therefore the thermal restrictions of *B.d.* have important implications for the host-parasite ecology of the disease and the effect of chytridiomycosis on wild amphibian populations (Kriger & Hero, 2006).

2.2.2 Host-Parasite Dynamics of *Batrachochytrium dendrobatidis*

B.dendrobatidis has the ability to affect susceptible hosts extremely variably. It has been observed that it is able to suppress populations down to extremely low levels, and may even be able to drive species to extinction. Mechanisms such as its low host specificity, broad host range and its ability to infect hosts at different life stages enable *B.d.* to have these devastating effects on its hosts.

A recent analysis from the Global Mapping Project has demonstrated that the presence of *B.d.* is significantly associated with environmental variables, such as temperature and

annual precipitation (Fisher *et al*, 2009). However *B.d.* experiences many physiological limitations outside of the host; many of which have been examined in an attempt to understand the dynamics of this fungus.

Experimental studies have shown that chemicals, such as Virkon and Ethanol were effective in causing 100% mortality of *B.d. in-vitro* (Johnson *et al*, 2003). The fungus is also highly sensitive to heat (37°C for 4 hrs), UV-B radiation and desiccation (> 3 hrs) (Johnson *et al*, 2003; Blaustein *et al*, 2003). White (2006) examined the hypothesis that exposing tadpoles to levels above 0.5% NaCl would inactivate and kill chytrid zoospores. However the continued exposure to high levels of salinity has shown to have negative impacts on the growth and survival of tadpoles (Wu & Kam, 2009; White, 2001). Wu & Kam (2009) examined the effects of salinity on the survival and development of tadpoles living in brackish water. More than 50% of tadpoles survived in 9ppt for over a month, with a few individuals tolerating 11ppt for 20 days. However tadpoles were shown to have lower survivorship, retarded growth and development at 9ppt. Tadpoles also metamorphosed earlier at a smaller size, as salinity increased.

2.3 Host – Parasite Dynamics

The foremost explanations for amphibian declines are often attributed to land use change, introduced species, over-exploitation, climate change, pollutants and pathogens (Collins *et al*, 2004). However although we have developed a comprehensive understanding of the ecological mechanisms with regards to land use change, introduced species and over-exploitation; our knowledge of climate change, pollutants and pathogens is often sparse (Collins & Storfer, 2003). These factors often involve complex and subtle mechanistic underpinnings, with probable interactions amongst multiple evolutionary and ecological variables (Collins & Storfer, 2003).

In an attempt to understand the dynamics of these interactions, scientists have devised several explanatory hypotheses; the novel pathogen hypothesis (NPH) and the endemic pathogen hypothesis (EPH). NPH suggests that novel strains are being introduced into geographic areas where they are encountering highly susceptible host species (Rachowicz *et al*, 2005). This spread has been attributed to the translocation of hosts, human encroachment, and through the introduction of exotic species. Conversely, the endemic pathogen hypothesis (EPH) implies that environmental disturbances suppress the immunological and ecological abilities of the host or parasite, altering the balance from a benign association towards a pathogenic, virulent relationship (Rachowicz *et al*, 2005). A key element in determining the EPH vs. NPH occurrence of chytridiomycosis will be a phylogenetic analysis of the patterns of genetic diversity harboured in the genome of *B.d.* (Fisher *et al*, 2009). It is currently unknown whether *B.d.* infecting Cumbrian natterjack populations is a novel strain or not, however preliminary genetic analysis of *B.d.* isolates globally show that geographically disparate isolates appear genetically similar, and that relatively little variation exists (Kilpatrick *et al*, 2009).

A combination of factors can affect the virulence levels of a pathogen (Lafferty & Gerber, 2002). High population densities facilitate transmission of pathogens, while exotic species may introduce novel strains into susceptible populations (Collins *et al*, 2004). Other factors which may enhance susceptibility include thermal stress from climate change, toxins from pollution, acidification, UV-B radiation (Kiesecker & Blaustein, 1995) and malnutrition (Beck & Levander, 2000).

Parasites often evolve and adapt in response to their selective environment and conditions. Highly virulence traits greatly comprise the host's immunity, and can aid the parasite in defeating a host's immune defence, which improves the likelihood of within-host persistence. In contrast, virulence may suppress host activity, thereby reducing the period of infectiousness (Collins *et al*, 2004).

Defining the effect on populations is often difficult, due to the unpredictable nature of population dynamics. However, epidemiology theories suggest that the transmission of disease increases exponentially with the density of susceptible hosts (Lafferty & Gerbert, 2002). This intensity suppresses the population below a threshold value, leading to reduced probability of transmission, increased probability of extinction of the pathogen and eventual recovery of the host population (Daszak *et al*, 1999). However this reduced capacity, can often lead the host exposed to stochastic events, and reproductive failure due to Allee effects (Lafferty & Gerber, 2002).

The understanding of what tips the balance from host-parasite coexistence, to host extinction can be unpredictable and is dependent on a number of related interactions (Collins *et al*, 2004). The interactions between virulence, susceptibility and population dynamics are represented diagrammatically in Figure 2.1 (Collins *et al*, 2004). These three elements ultimately determine whether species fall within the coexistence or extinction continuum.

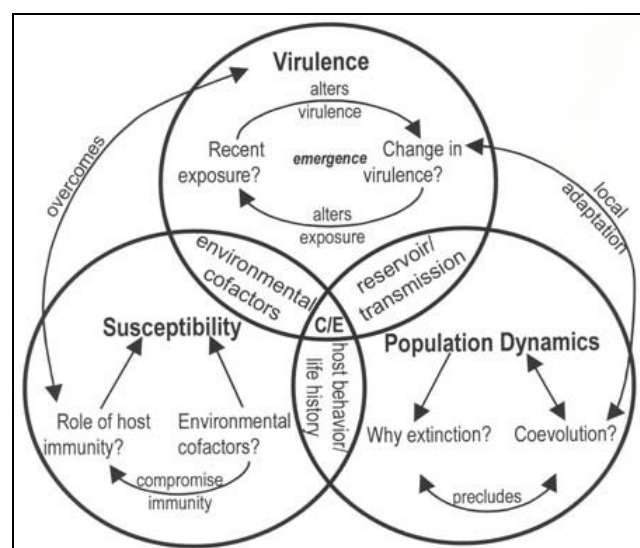


Figure 2.1. Interactions amongst virulence, susceptibility, and population dynamics (Collins *et al*, 2004).

2.3.1 Coevolutionary dynamics of host-parasite interactions

The coevolution of parasite and host represents a delicate relationship, where the needs of the parasite are considered against the needs of the host, in order to maintain continual existence. This relationship can often be highly influenced by variations in environmental factors and resources.

Research by Boots (2011) examined the evolution and coexistence of a hosts defence against parasites, and assessed how the hosts resistance and associated costs depend on resource availability. They made a convincing argument that the level of trade-offs and resistance were correlated with the resources present in the environment, implying that different resistance mechanisms may evolve within different environments. This was demonstrated by the higher rate of extinction in poorer-quality environments where there was a higher susceptibility to infection and a relative inability to evolve resistance (Boots, 2011). Therefore there is potentially a negative feedback where a suppressed population which is at a higher risk of extinction due to poor resources also comprises individuals that evolve to be more susceptible to disease (Boots, 2011).

Environmental factors are also known to affect the strength and specificity of interactions between hosts and parasites. Mostowy & Engelstadter (2011) investigated the effect of environmental change on host-parasite coevolutionary outcomes. They showed that these environmental factors can have a substantial effect on the antagonistic host-parasite interaction balance, by affecting the selective pressure and the level of specialism within a host population. A similar study by Wolinska & King (2009) also examined the ability of temperature to affect the host-parasite interaction. They suggested that this environmental variable can greatly affect host biochemical, physiological and behavioural processes, which can suppress the host's resistance mechanism.

2.4 Environmental co-factors

Many aspects of the host-pathogen relationship, are influenced by environmental factors such as salinity and pH, and will eventually be expressed as heterogeneities in the presence and prevalence of infection (Fisher *et al*, 2009).

The role of climate change in driving amphibian declines has yet to be systemically addressed (Pounds, 2001). However evidence is now providing a compelling argument that climatic fluctuations may have significant effects on amphibian populations by altering local environmental conditions, which in turn affects host fitness (Carey & Alexander, 2003; Reading, 2007). Reading (2007) found substantial evidence of correlations between the onset of warmer than average years, and declines in survival rates. There was also marked declines in the body conditions of common toads (*Bufo bufo*) during warmer years (Reading, 2010). Rohr & Raffel (2010) tested the hypothesis that increased regional variability could account for spatiotemporal patterns of *Atelopus* declines, associated with *B.d.* They found significant evidence that the climate variability hypothesis was a strong predictor of *Atelopus* declines, consistent with previous findings that temperature variability

compromises amphibian immune defences and that disease outbreaks frequently occur during extreme temperature events (Martin *et al*, 2010). Furthermore research by Bosch *et al* (2007) analysed long-term climatic data to investigate the link between climate change and the onset and persistence of chytridiomycosis. Significantly they found that changes in circulation patterns have increased climate variability and elevated yearly average temperatures, which are associated with the occurrence of chytrid related disease, consistent with the chytrid thermal optimum hypothesis. However it is important to consider that chytrid mortality events are probably the result of a complex web of interactions, and the effects of climate change will be dependent on other factors such as host density, community composition, and competitors (Bosch *et al*, 2007).

Environmental factors can increase parasite mortality and suppress parasite infectivity. Perrigault *et al* (2010) tested the effect of environmental parameters on the survival and growth of quahog parasite *in-vitro*. They found that parasites could tolerate extreme temperatures, and were able to grow under the tested range of salinities (22-37 ppt), with only salinity levels as low as 15ppt appearing to inhibit parasite growth. However combined effects of salinity and temperature had a marked effect, with reduced survival occurring in seawater at high temperatures (25-35ppt, 23°C). Research by Studer *et al* (2010) examined the effects of temperature on an intertidal parasite. They found that the survival rate and infectivity decreased steadily with an increase in temperature. This suggests that the rise in temperature is directly affecting the fitness of the parasite, leading to a faster depletion of their energy resources.

Research by Koprivnikar *et al* (2006) found a marked correlation between the reduced prevalence of trematode infection in frog tadpoles and the presence of pond eutrophication.

It is possible that the prevalence and presence of disease could be triggered by various environment co-factors (Blaustein & Kiesecker, 2002). For instance, a complex web of changes in precipitation, reduced water levels, UV-B radiation and contaminants has been hypothesised to have played a role in chytrid outbreaks in Costa Rica (Lips, 1998; Pounds & Crump, 1994). Furthermore shifts in water pH in Spain, have been implicated as a stress factor that enhanced the susceptibility of hosts to chytrid outbreaks (Bosch *et al*, 2001; Bosch *et al*, 2007). Research by Santaliestra *et al* (2011) examined the potential effect of environmental UV-B on the susceptibility of larvae to *B.d.* and found that the prevalence of *B.d.* was significantly lower in tadpoles exposed to environmental UV-B intensities. This suggests that the interaction between UV-B and *B.d.* may be antagonistic, rather than synergistic, which highlights the importance of considering multiple stressors when analysing the ecological responses of amphibians to *B.d* (Santaliestra *et al*, 2011).

Amphibians, like other organisms, encounter a wide range of environmental co-factors with resulting effects that are often complex, as demonstrated in Figure 2.2.

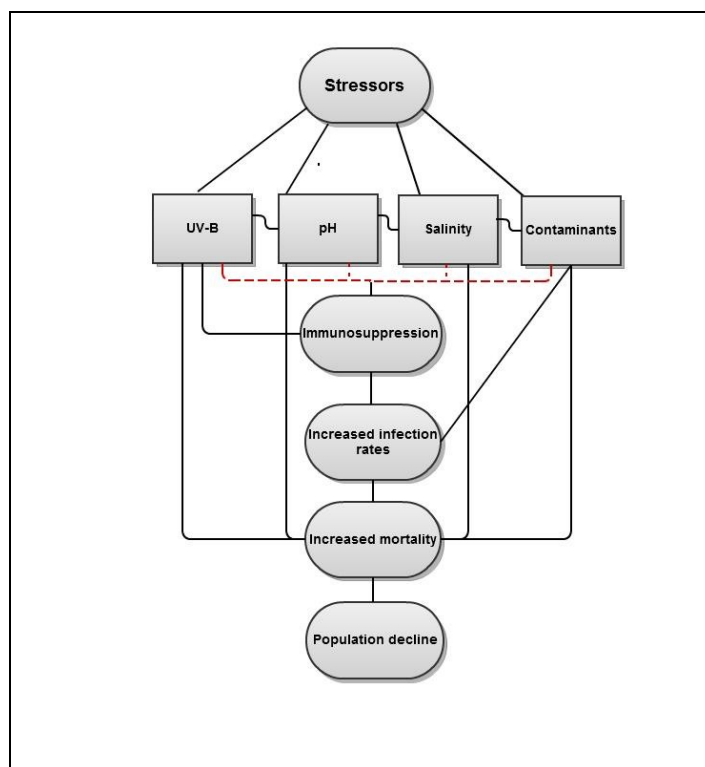


Figure 2.2 Complex interactions among abiotic and biotic factors influence amphibian populations. Amphibian populations are subjected simultaneously to stressors like salinity and pH that interact in a dynamic fashion, and can cause sub-lethal and lethal effects (adapted from Blaustein *et al*, 2003).

Although some populations are undoubtedly affected by such interactions, individual life history traits will characterise the population's ability to respond to these abiotic and biotic factors.

2.4.1 Salinity and pH

Alterations of abiotic factors are predicted to impact disease dynamics, particularly for pathogens with complex life cycles involving free-living infectious stages, like chytridiomycosis and inter-tidal trematode parasites (Koprivnikar *et al*, 2010).

Chytrids are affected by a number of different physical and biological environmental factors, which impede their growth and impact their population composition. The physical factors include primarily salinity, pH, dissolved oxygen concentration and temperature (Gleason *et al*, 2008).

Although some species have been observed growing in saline estuaries, most freshwater and soil chytrids are unable to grow effectively in undiluted sea water (Gleason *et al*, 2006). Instead they prefer environments with low osmotic potentials (Gleason *et al*, 2006). Therefore chytrids which have evolved in estuarine environments are able to tolerate a much broader range of NaCl concentrations than freshwater chytrids. Several laboratory studies have supported this finding. For example, Chukanhom & Hatai (2004) found that freshwater and soil chytrids grew on media supplemented with 1% NaCl, but not with 2% or over NaCl. All isolates also grew at a wide range of pH and the optimum pH for growth was

6.0-8.0. A similar study by Gleason *et al* (2006) also observed very similar reactions when exposing chytrids to NaCl. Also chytrid isolated from Cumbrian natterjack toads grew faster in media with a salinity of 0.4%, but wasn't able to grow above 0.9% (Minting, pers. comm).

Amon and Arthur (1981) looked at the effects of ion concentrations on chytrid growth, and found that chytrid required higher levels of Na⁺, Mg⁺ and Ca⁺ than those found in freshwater, but lower than those in seawater for optimum growth.

Research by Gleason *et al* (2010_a) has suggested that zoospore fungi have adapted to stressful or extreme environments where the ranges of abiotic parameters are in excess of the normal values for optimum growth and survival. Gleason *et al* (2010_b) tested this hypothesis by examining the growth, and survival of zoospore fungi to extreme levels of pH. All isolates grew rapidly in growth media with pH values near neutral. Most isolates could be maintained at pH 4.7 and pH 8.9, with one growing down to pH 2.9, four down to pH 3.3 and four up to pH 11.2. This suggests that zoospore fungi are able to tolerate a wide range of pH in a stable state, thus explaining their presence in hostile environments. Furthermore a few species of zoosporic true fungi such as isolates of *Thalassochytrium* (Nyvall *et al*, 1999) and *Chytridium* (Muller *et al*, 1999) have adapted for growth as parasites in marine environments.

Research by Piotrowski *et al* (2004) demonstrated that buffers affected the growth of *B.d.* For instance, the fungus grew poorly at a slightly acidic pH of 6. However its zoospores have been observed to grow at this level, and once inside the host the fungus may be buffered from external conditions affecting the pH further. Nonetheless this highlights some interesting physiological requirements of *B.d.* pH tolerances.

Similar responses to interactions with salinity and pH have also been observed with other bacterium and viruses in different species (Stallknecht *et al*, 1990; Brown *et al*, 2008; Lei & Poulin, 2011).

For instance, strong interactions have affected the infectivity and growth of avian influenza aquatic viruses. Research by Stallknecht *et al* (1990) found that pH and salinity had a marked synergistic effect on the viruses' growth. The virus was stable at ranges from neutral to pH 8.5; however infectivity decreased rapidly below levels of 6.0. However interestingly under saline conditions, infectivity was inversely related to salt content, and optimum pH tolerance shifted to more acidic conditions. Brown *et al* (2009) conducted a similar study and established that avian influenza were most stable at pH levels of 7.4-8.2, and in fresh to brackish salinities (0 – 20,000 parts per million (ppm)). On the other hand the virus had a stunted persistence in acidic conditions (pH < 6.6) and at high salinity (>25,000 ppm).

An effect of salinity on reproduction has also been examined in intertidal trematode parasites. Research by Lei & Poulin (2011) found that long-term exposure to salinity caused substantial decreases in parasite replication and its survival outside the host. Therefore this

indicates that estuaries may act as sinks, and mudflats outside estuaries may act as sources, or foci in the transmission dynamics of the parasite (Lei & Poulin, 2011). Similar research by Koprivnikar *et al* (2010) investigated the response of trematode parasites to altered temperature, salinity and pH. They found that one parasite fared best at the higher salinity (40ppt) whereas the other reacted negatively. On the other hand pH caused no remarkable effect. These results may reflect different physiological requirements of the two species, and demonstrate that parasitism in intertidal zones may be impacted by alterations in the environment dynamics (Koprivnikar *et al*, 2010).

An effect of salinity and seasonal variability on the prevalence of enteric pathogens in a subtropical estuary was observed by Lipp *et al* (2001). They found that lower salinity due to wet weather events, in association with low winter water temperatures significantly enhanced the presence of enteroviruses. A similar study by Ferguson *et al* (1996) observed the lowest concentrations of enteric viruses at sites which had a higher saline level.

Salinity was found to be significantly related to the abundance of sea lice populations on estuarine sea trout. Research by Heuch *et al* (2002) noticed that sea lice populations declined by 10% after prolonged periods of low salinity, and observed that lice populations experience frequent bottlenecks during this period.

The infectivity and prevalence of marine endoparasites are also affected by varying levels of salinity. Research by Moller (1978) looked specifically at the effects of salinity and temperature on the development and survival of fish parasites. The endoparasites were found to be significantly affected by high levels of salinity, with growth entirely suppressed between 7-20‰ salinity.

Although there appears to be some isolates which can tolerate higher levels of salinity (Nyvall *et al*, 1999; Muller *et al*, 1999) general patterns indicate that the majority of zoosporic fungi are unable to grow in high osmotic environments. Trends in the studies reviewed also appear to indicate a correlation between the suppressed growth and survival of parasites, and higher salinity levels.

2.5 rt-PCR Inhibition

Diagnosis of the chytrid fungus, *Batrachochytrium dendrobatidis* has historically relied on histological examination, eosin staining of toe clippings or skin scrapings (Daszak *et al*, 1999). However this technique is often invasive and insensitive particularly at early stages when treatment may be available (Boyle *et al*, 2004). Immunohistochemistry has also been used as an effective diagnostic detection tool, which provides distinct visualisation of *B.d.* (Ells *et al*, 2003).

Advancements in molecular technologies have led to the development of real-time TaqMan assays which are ideal for diagnostics due to their ability to rapidly and sensitively detect target DNA (Boyle *et al*, 2004).

However rt-PCR can be inhibited by components of biological samples and organic detritus which inhibit the amplification of DNA by polymerases. The inhibition can be total or partial, and can present as a reaction failure or as reduced sensitivity in detection (Davalieva & Efremov, 2010). The inhibitors generally act at one or more of three essential points in the following ways; they interfere with the cell lysis necessary for extraction of DNA, they interfere by nucleic acid degradation and they inhibit polymerase activity for the amplification of the target DNA (Al-Soud & Radstrom, 1998).

The environmental factors pH and salinity were investigated within this study as potential rt-PCR inhibitors, which would interfere by inhibiting polymerase activity for the amplification of target DNA.

Davalieva & Efremov (2010) examined the effects of Na⁺, K⁺ and Mg²⁺ ions, and found that Na⁺ ions were more inhibitory (over 40Mm) than K⁺ ions (over 80Mm). Furthermore the inhibitory effect of divalent ions (Mg²⁺) was more pronounced than that of monovalent ions (Na⁺ and K⁺). A similar study by Al-Soud & Radstrom (1998) demonstrated similar results, and found that the polymerase Taq was particularly sensitive to Ca ions.

These inhibitions can be reduced or eliminated through the use of another more resistant thermostable DNA polymerase (Davalieva & Efremov, 2010), by diluting the inhibited samples (Wilson, 1997), through ensuring the reaction conditions and settings are optimised (Wilson, 1997) and by adding additional reagents (Kreader, 1996).

Kreader (1996) evaluated the benefits of adding Bovine Serum Albumin (BSA) to PCR to relieve amplification inhibition and found varied effects. Whilst humic acids, tannic acids, or extracts from feces, freshwater or marine water were accommodated in PCR when BSA was used (400ng of BSA per µl) no significant effect was observed when minimum inhibitory levels of bile salts, bilirubin, or NaCl were present. Furthermore a study conducted by Plante *et al* (2010) found that by incorporating BSA into a rt-PCR kit, greater sensitivity was exhibited in the presence of large quantities of PCR inhibitors for many different primer/probe combinations.

Elsewhere studies have observed different degrees of rt-PCR inhibition. Walker *et al* (2007) used IPCs to test for the evidence of inhibition in water and sediment samples. They found no identifiable inhibition within the water samples; however 2 out of 6 of the sediment samples showed evidence of significant inhibition. Garner *et al* (2009) also used IPCs to test for inhibition in the presence of *B.d.* within experimental and field samples. They observed no evidence for PCR inhibition in any of the experimental samples. However within the field samples PCR inhibition was present in 9 out of the 29 DNA extractions.

2.6 Study Species (Natterjack toad, *Epidalea calamita*)



Figure 2.3 Picture of an adult *E. calamita* (ARG, 2010).

The natterjack toad, *Epidalea calamita*, is an endangered species in Britain and has been legally protected since 1975. It's distributed throughout western and south-west Europe, with the UK encompassing the north-western edge of its range, isolated from the mainland populations since around 8000 BP (Hitchings & Beebee, 1996).

The natterjack toad has suffered dramatic declines since the first half of the twentieth century, due partly to habitat destruction but mostly to successional changes in its specialised biotopes and anthropogenic acidification of breeding sites (Denton *et al*, 1997). For instance, two large ponds previously used in Woolmer Forest have been deserted in the last fifty years, as they are now too acidic (pH <4.5) to support embryonic and larval development (Beebee, 1990).

The current known distribution of *E. calamita* is restricted to several sites in South and East England, North West England and South West Scotland, as shown in Fig 2.4. Many existing populations have become small and fragmented, where previously they existed as part of larger populations (Beebee, 1990).

In the UK, *E. calamita* is predominantly found in coastal dune settings, lowland heaths and upper saltmarshes (Beebee, 1990). In spring, they rely on a network of ephemeral breeding ponds, where larvae can metamorphose within eight weeks (Beebee, 1990).

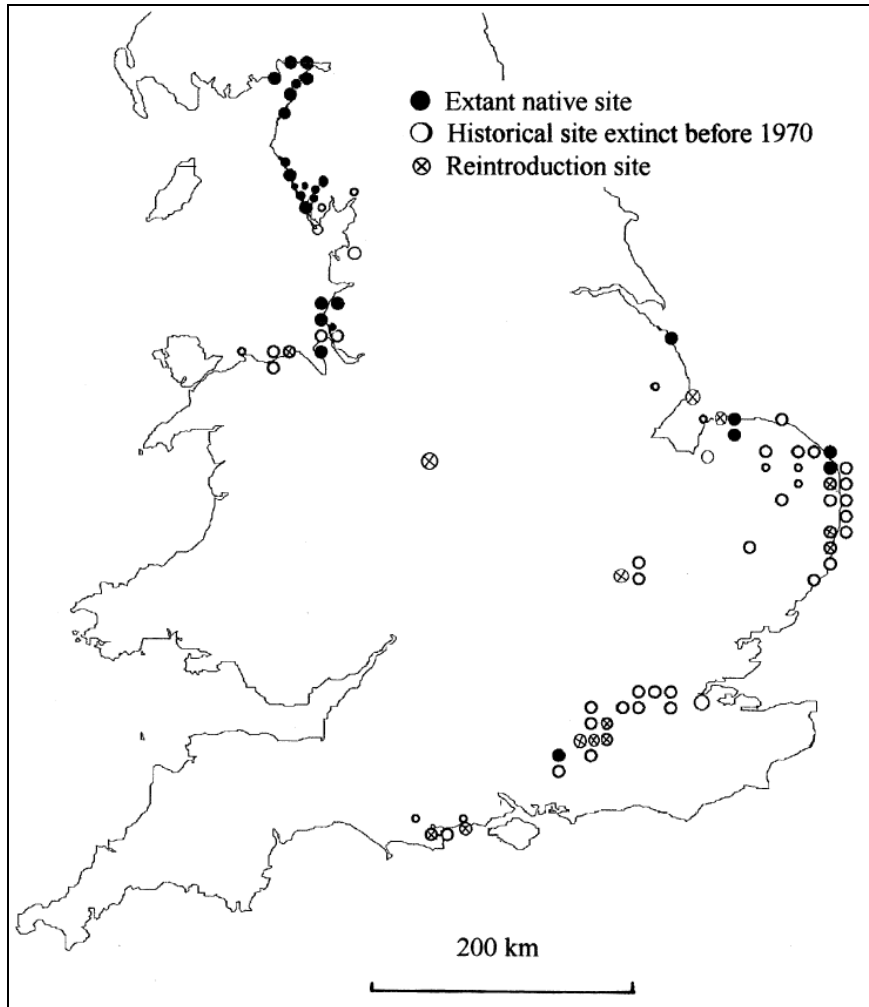


Figure 2.4. Map of *E. calamita* distribution in the UK. (Denton et al, 1997).

Management of heath and dune habitats have focused on the restoration and maintenance of seral succession, through clearance of invasive scrub and woodland vegetation. Other conservation actions such as translocation and reintroduction have been carried out in an attempt to expand current dwindling populations (Denton *et al*, 1997).

3. Materials and Methods

3.1 Methodology Framework

All surveys were carried out in the spring season, at known *E.calamita* sites in North West England and Southern England. They were designed to be carried out at this time, as this is when amphibians aggregate at breeding sites, which optimises the potential of capturing meaningful sample sizes. At each site, the aim was to collect 25 - 30 adult individuals of *E.calamita*. Thirty individuals was the standard procedure set for the UK Chytrid surveillance, as it gives 99% confidence to detect 15% prevalence of infection (DiGiacomo & Koepsell, 1986).

Sites were identified through literature reviews, and through the assistance of ZSL and the Amphibian and Reptile Conservation Trust. The Natterjack Toad Site Register for the UK (Beebee & Buckley, 2001) was used to determine the location of the ponds.

Swab sampling for real-time PCR using the TaqMan assay (Boyle *et al*, 2004) was set as a protocol for the UK Chytrid surveillance survey. This swabbing technique is non-invasive and easy to use.

Conductivity and pH measurements were recorded on-site, at the time of sampling to prevent against any degradation effects. Water samples were also collected, and analysed for salinity in the laboratory. This has, in combination with conductivity readings, provided an accurate measurement of pond water salinity at the time of sampling.

E.calamita sites were classified as locations which encompassed singular or multiple numbers of ponds. Ponds were defined as a body of standing water, either natural or man-made, where *E.calamita* were residing, or had resided historically. Site and pond locations were obtained from The Natterjack Toad Site Register for the UK (Beebee & Buckley, 2001).

The purpose of this research was to investigate the apparent pattern of *B.d.* scores in relation to salinity and pH, and to determine the prevalence of *B.d.* at the surveyed sites.

3.2 Fieldwork/Sampling

3.2.1 Study Site

Surveillance surveys were undertaken at known *E.calamita* sites in North West and Southern England (Fig 3.1) as identified through literature reviews, and through the assistance of ZSL and the Amphibian and Reptile Conservation Trust.

Due to budget and time constraints, the aim was to survey 10 -20 sites, many of which had multiple ponds. If we were unable to locate any *E.calamita* on the first visit, the sites were visited for a second time. Existing data which has been collected from five *E.calamita* sites in Cumbria since 2008 was also made available for this study (Minting, pers. comm).

All pond locations were recorded using a GPS device, and the coordinates were used to conduct spatial comparisons.

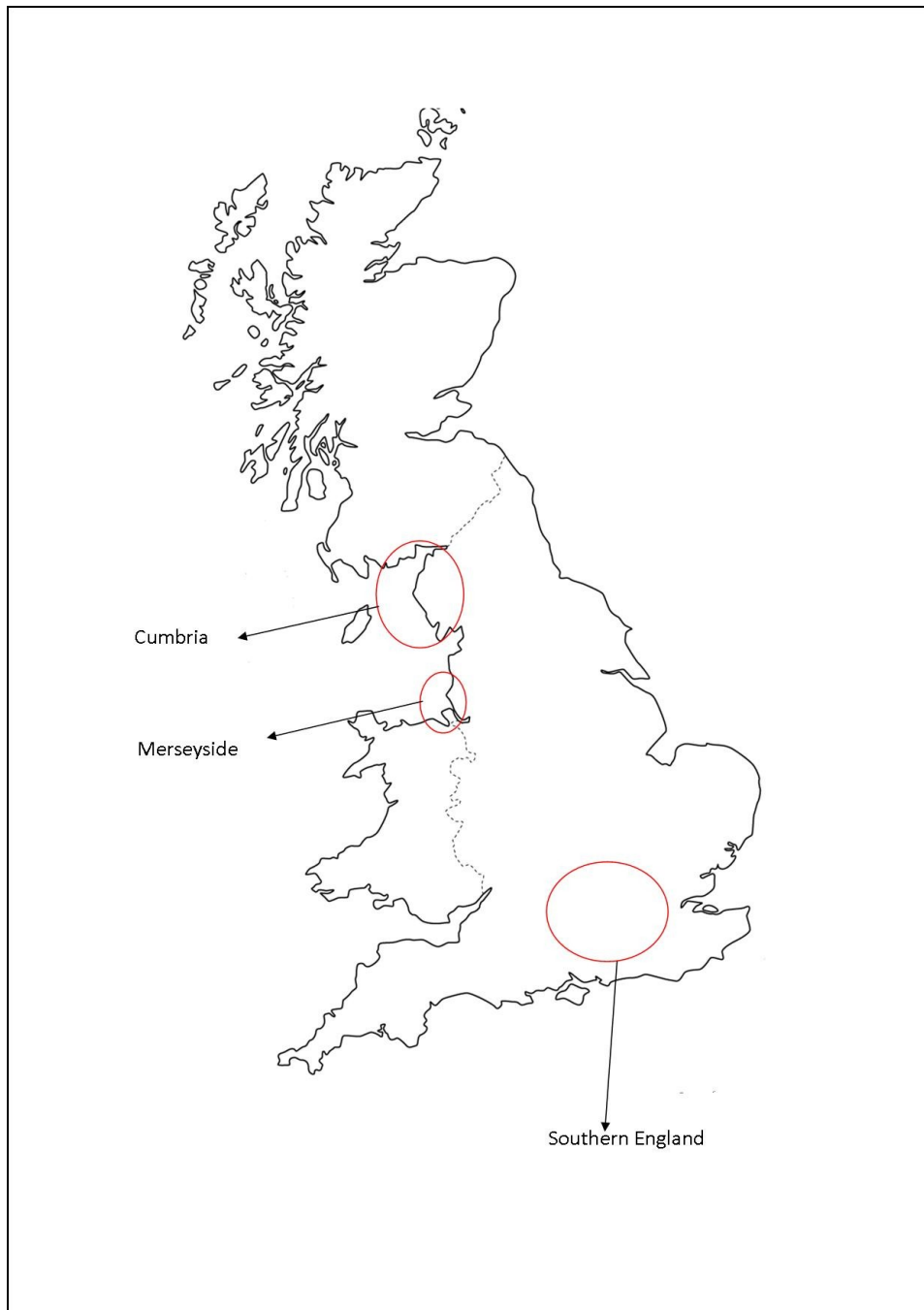


Figure 3.1 Map showing *E.calamita* regions sampled for *B.d.* surveillance

3.2.2 Field methods

Surveys were carried out after dark using a Clulite Torch and head torch, in accordance with the peak hours of *E.calamita* activity. As a precautionary measure and to minimise the likelihood of cross-contamination, visits were restricted to one site per evening.

To prevent spreading *B.d.* infection, all the equipment used (wellingtons, buckets, waterproofs) was thoroughly disinfected using Virkon (1% concentration) prior to visiting different sites. Also fresh gloves and swabs were worn when sampling individual toads.

All toads were captured by hand. These were then placed into a plastic bucket containing pond water until we were happy that all of the toads had been captured. Prior to swabbing all toads were rinsed with freshwater to limit any inhibition from organic detritus or brackish water.

Sterile cotton swabs were used for the swab sampling. Abdominal and hind leg swabs were taken from each individual, and then details of the animal were recorded (i.e. species, sex and any other additional abnormal information, such as signs of deformity or infection). The animals were then immediately released back into their original pond.

Sterile 50ml plastic containers with secure lids (MediPost, UK) were used to collect water samples from each *E.calamita* pond. Conductivity (Imperial College, UK) and pH (ATP, UK) devices were used to take measurements at each site. The pH meter was calibrated (pH 4.01; pH 7.01 Buffer solution; ATP, UK) after every 10 uses as recommended by the manufacturer.

Once collected, all samples were maintained in a cool, dry environment until they were transported to the laboratory at IoZ for analysis.

3.3 Laboratory methods

To avoid cross contamination of the samples, which may result in false positives, precautionary measures were taken at all stages. Clean gloves and a lab coat were constantly worn, and sterile Eppendorf tubes and beads were used. Fresh cutting blades, pipette tips and sterile Petri dishes were used each time different samples and chemicals were handled. The surface of the fume cabinet was carefully disinfected with ethanol between each use.

3.3.1 Extraction of *B.d.* DNA

The extraction of *B.d.* DNA was carried out according to the Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity (RACE) Protocol.

For each sample, a 1.5ml Eppendorf centrifuge tube was prepared with 0.03 – 0.04g of 0.5mm Zirconium silica microbeads and 60µl of Prepman Ultra solution. The swabs were then moved to the fume cupboard, where the tip of each was cut off using a sterile Petri dish and scalpel, and placed into the prepared centrifuge tube. The contents of the samples were then homogenised using a Bead Beater for 45 seconds, followed by a centrifuge for 30 seconds (speed 14500rpm). After repeating the process of homogenisation and centrifugation, the samples were placed in a 100°C water bath for 10 minutes and then removed to cool. The samples were then centrifuged for a further 3 minutes at 14500rpm, before the supernatant was pipetted into a sterile 1.5ml Eppendorf. The supernatant was

then diluted at a rate of 1:10, to account for any inhibition in the Prepman solution. 36µl of sterile water was initially pipetted into a sterile 1.5ml Eppendorf; thereafter 4µl of supernatant was pipetted and mixed well. All extractions were then stored in labelled 1.5ml Eppendorf tubes at -17°C.

3.3.2 Real-Time PCR (rt-PCR)

To determine the presence of *B.d.* and quantify the level of infection, real-time polymerase chain reaction (Rt-PCR) using a TaqMan assay (Boyle *et al*, 2004) was used.

The Real-Time PCR analysis was carried out according to the Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity (RACE) Protocol.

PCR Master Mix containing ITS1-3 and 5.85 DNA primers, Chytr MGB2 probe, TaqMan, and H₂O was prepared according to the RACE protocol. To ensure reliability, all samples were run in duplicates. The PCR Master Mix (20µl) was put in each well of a 96 well microtitre plate (Applied Biosystems, USA) together with the DNA dilution (5µl). A negative control and four standards (containing 100, 10, 1 and 0.1 genome equivalents of *B.d.* zoospore DNA) were placed in each plate to obtain genomic equivalents (GE) for potential positives. A clear PCR seal was fitted onto the 96 well plate, and centrifuged at 4000rpm, 12°C for 3 minutes.

The plate was then placed in the PCR Real-Time 7300 System (Applied Biosystems, USA), where the amplification cycle was set as; Stage 1: 50°C for 2 minutes, Stage 2: 95°C for 10 minutes, Stage 3: 95°C at 15 seconds and then 60°C for one minute (repeated 60 times). The Rt-PCR system software calculated genomic equivalents (GE) of *B.d.* zoospores for positive samples from the Ct number, which is the point where the amplification plot crosses the threshold. As the sample extractions were diluted 1/10 before PCR, 0.1 GE was considered as the minimum acceptable value for positive infection.

3.3.3 Internal Positive Controls (IPC)

TaqMan Exogenous IPCs were used to monitor the PCR process and ensure that a negative result is not generated due to a failed PCR. This technique distinguishes true target negatives from PCR inhibition and is especially important to include when testing samples that may have picked up quantities of organic detritus or may be affected by salt inhibition. The procedure involves spiking a known concentration of target DNA (50x IPC DNA) into one of each duplicate well. Samples with Ct values significantly higher than a control were considered to be influenced by PCR inhibition. Using an IPC in a new site can reassure you that you are determining the true uninhibited prevalence and intensity of infection.

IPC Master Mix containing ITS1-3 and 5.85 DNA primers, Chytr MGB2 probe, TaqMan, 10x Exo IPC mix, 50x Exo IPC DNA, and H₂O was prepared according to the RACE Protocol.

The IPC Master Mix (20µl) was put in one of each duplicate well of a 96 well microtitre plate (Applied Biosystems, USA) together with the DNA dilution (5µl) and the negative and standards.

The plate was then placed in the PCR Real-Time 7300 System following the same cycle as with the Real-Time PCR (rt-PCR) protocol.

The optimum Ct value for IPC DNA amplification is between 35 – 37Ct. Therefore any amplification higher than 40Ct was defined as partially inhibited, with 50Ct and above defined as totally inhibited.

3.3.4 Bovine Serum Albumin (BSA) and Environmental TaqMan

In order to limit the inhibition defined by IPC, Bovine Serum Albumin (BSA) and Environmental TaqMan were used as additives for their ability to neutralise inhibition.

Bovine Serum Albumin (BSA) has widely been used as a stabilising and blocking agent and is a common additive for PCR amplifications (Kreader, 1996).

The BSA solution used contains 0.05g of BSA in 1ml of sterile water. 20µl of BSA solution was added into the Master Mix recipe. To compensate for the extra volume, the H₂O quantity was reduced by 20µl. The Master Mix including BSA was then put in each well of a 96 well microtitre plate together with the DNA dilution (5µl).

Environmental TaqMan (Applied Biosystems, UK), was used as an alternative reagent. It is able to provide reliable detection of pathogens, even in the presence of contaminants such as organic detritus and salt.

Environmental TaqMan was used in place of the TaqMan Universal PCR MasterMix. All of the other components and volumes remained the same. In order to detect whether the Environmental TaqMan was effective in neutralising any inhibition, the IPC MasterMix was added to one of each duplicate well of the 96 microtitre plate.

3.3.5 Chemical Titration

Chemical titration was used as an analytical method to determine the quantities of salinity within our water samples.

A silver nitrate solution containing 5g of AgNO₃ and 1000ml of distilled water was used to precipitate chloride in a known volume of artificial seawater. Potassium dichromate K₂Cr₂O₇, was used for its abilities to act as an indicator. The potassium dichromate remains colourless as long as there is Cl⁻ present, but the instant the last of the Cl⁻ is bound up as silver chloride, the solution turns orange.

Using a graduated pipette 5ml of the sample was transferred into a 125ml Erlenmeyer flask. The potassium dichromate (5ml) was then added into the same flask. A magnetic stirrer was placed in the flask and set under the burette on a stirring plate.

The 50ml burette was then filled with AgNO₃ solution. The AgNO₃ solution was slowly added from the burette until an abrupt colour change from milky yellow to orange was detected. The amount of AgNO₃ used was then recorded.

The unknown salinity was calculated using the following equation; unknown salinity = ml AgNO₃ (unknown)/ml AgNO₃ (known) * known salinity.

3.4 Data Analysis

3.4.1 Data arrangement

The final rt-PCR and IPC results were gathered in a Microsoft Excel spread sheet together with the *B.d.* results produced by Peter Minting from 2009 until 2011. Conductivity, pH and chemical titration results were also compiled within a spread sheet format.

3.4.2 Statistical analysis

All of the following statistical analyses were carried out using program R (The R Foundation for Statistical Computing, R version 2.11.1., <http://www.r-project.org>)

For all analyses, a confidence level of 95% was used to infer any significant impact. An example of the code used for statistical analysis in R, has been included in Appendix III.

For this study, the data set was analysed in the following ways;

1. The prevalence of positive versus negative samples was analysed against the total sample size across each individual site, using a binomial two-tailed test.
2. A binomial two proportion test was used to analyse the positive samples against the total sample size between saline and freshwater samples. Saline and freshwater sites were defined by their conductivity and titration readings, as measures of salinity.
3. An unpaired T-test was used to analyse the genomic equivalent (GE) scores between the saline and freshwater samples.
4. GLMs analysis for proportional data (Crawley, 2005) was carried out to test the observed *B.d.* infection level (infected/uninfected) against “salinity” and “pH” as continuous explanatory variables.
5. Co-variance analysis for continuous variables was used to examine the relationship between “electrical conductivity” and “titration” for salinity, and “salinity” and “pH”.
6. GLMs analysis for proportional data (Crawley, 2005) was used to test for any regional patterns. “Latitude” was used to measure the geographical location of each site. This was then analysed against the “Prevalence” of infection at each location.
7. Data from sites 2, 4, 7, 8, 9, 10 which consisted of a selection of freshwater and saline sites with high conductivity readings and/or low repeat positives, were analysed using IPC to test for any inhibition. Inhibition was determined by the level

of the count types (Ct). GLM analysis was then used to test for any significant inhibitory effect.

8. GLMs analysis for proportional data (Crawley, 2005) was used to test for any differences between the prevalence of *B.d.* before and after the addition of Environmental TaqMan.
9. A paired T-test was carried out to compare the genomic equivalent (GE) scores of each positive sample before and after Environmental TaqMan was added. Scores refer to the amount of *B.d.* genome zoospore DNA present within each sample.

4. Results

A confidence level of 95% (p - value <0.05) was used to infer any significant statistical association. Any relationships which proved to be particularly significant were then referred to at a 99% confidence level (p – value <0.01).

4.1 rt-PCR results

4.1.1 Cumbrian rt-PCR results

The rt-PCR results of 10 coastal natterjack sites located throughout Cumbria are displayed in Table 4.1. *B.d.* was detected at 7 of the sites sampled, with low rt-PCR repeats also presenting at 6 of these sites. *B.d.* was not initially detected from samples at Haverigg, Soutergate and Subberthwaite.

Site Number	Location	PCR Results (Infected/Total) *	Low Repeats **	B.d. infection (+/-)
1	Braystones	1/8	1/8	+
2	Drigg	5/30	15/30	+
3	Annaside	1/6	1/6	+
4	Haverigg	0/26	0/26	-
5	Soutergate	0/5	0/5	-
6	Sandscale	21/42	6/42	+
7	Dunnerholme	4/30	2/30	+
8	Sandside	3/25	4/25	+
9	Sharp Street	2/8	0/8	+
10	Subberthwaite	0/1	0/1	-

Table 4.1 *B.d.* rt-PCR results from Cumbrian sites (“Red” = Positive *B.d.* sites)

* PCR results are shown as number of infected individuals/total sampled at each site

** Low PCR results which have only amplified within one well. (In addition to true positives where amplification occurs in both wells)

The rt-PCR results from 4 coastal natterjack sites, which have been monitored annually as part of an on-going study, are displayed in Table 4.2. The data collected in 2008 at Grune, was part of the nationwide ZSL National Chytrid survey. Since the respective surveys commenced, positive results have been detected at all sites, except from Anthorn.

Site Number	Location	PCR Results (Infected/Total) *				B.d.infection (+/-)
		2008	2009	2010	2011	
11	Bowness	-	3/43	1/32	2/27	+
12	Grune	0/21	30/102	17/69	16/64	+
13	Mawbray	-	11/56	29/76	38/60	+
14	Anthorn	-	0/11	0/35	0/16	-

Table 4.2. *B.d.* rt-PCR results from 4 Cumbrian sites between 2008 – 2011 (ZSL National Chytrid Survey 2008; on-going PHD research, P.Minting) (“Red” = Positive *B.d.* sites)

* PCR results are shown as number of infected individuals/total sampled at each site

4.1.2 Merseyside and Southern England rt-PCR results

The rt-PCR results from 3 natterjack sites located within Merseyside and Southern England are shown in Table 4.3. *B.d.* was detected at two out of the three sites sampled.

Site Number	Location	PCR Results (Infected/Total) *	Low Repeats **	B.d. infection (+/-)
15	Ainsdale	4/30	5/30	+
16	Frensham	0/2	0/2	-
17	Sandy	2/5	0/5	+

Table 4.3. *B.d.* rt-PCR results from Merseyside (Ainsdale) and Southern sites (Frensham & Sandy) ("Red" = Positive *B.d.* sites)

* PCR results are shown as number of infected individuals/total sampled at each site

** Low PCR results which have only amplified within one well (In addition to true positives where amplification occurs in both wells)

The location and infection status of all 17 *E.calamita* sites surveyed for *B.d.* are illustrated in Figure 4.1.

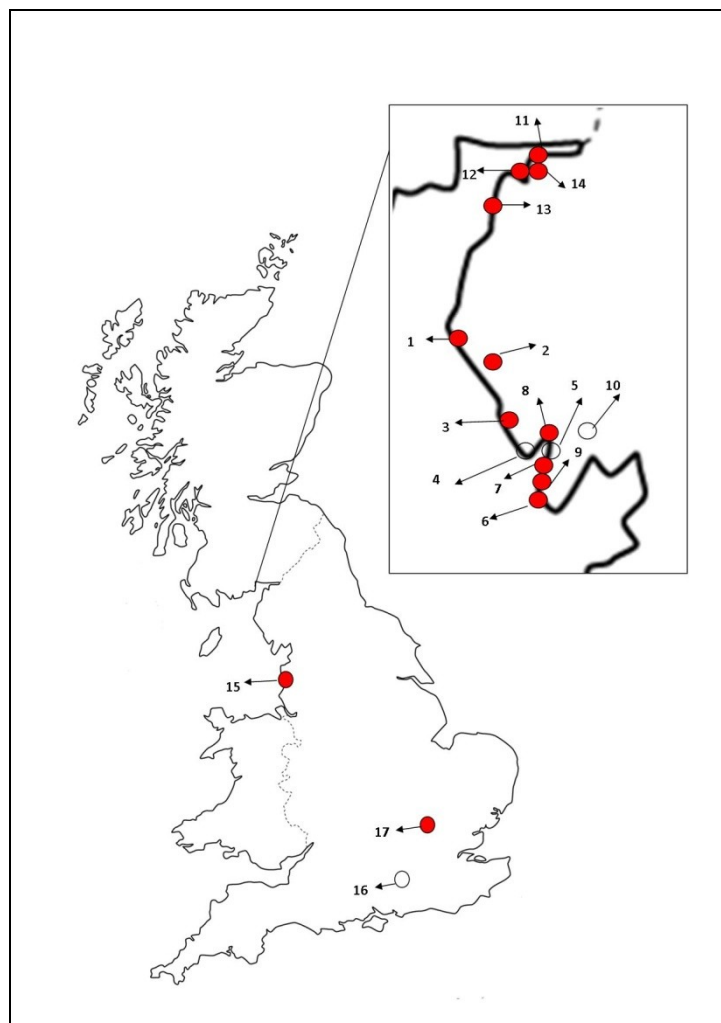


Figure 4.1. Infection status of *E.calamita* sites. Numbers refer to sites as detailed in Table 4.1 – 4.3.

● *B.d.* infected sites, ○ No *B.d.* detected sites.

4.2 Salinity and pH

4.2.1 Electrical Conductivity and Chemical Titration

Electrical conductivity and chemical titration were used as measures of salinity at each pond where swabs were collected. pH measurements were also recorded at each site.

The salinity and pH results across all sites are presented in Table 4.4. The ranges indicate that there was a variation between salinity and pH across the multiple ponds. Appendix II contains the salinity and pH readings from each pond within each site.

Site Number	Location	Conductivity(uS)	Titration	pH	B.d. infection (+/-)
1	Braystones	164 – 173	0.10 – 0.16	8.18 – 9.42	+
2	Drigg	122 405	0.09 – 0.26	7.32 – 7.81	+
3	Annaside	275	0.20	7.64 – 8.29	-
4	Haverigg	562 – 11610	0.13 – 0.59	8.03	-
5	Soutergate	4860	0.20	7.08	-
6	Sandscale	426 – 863	0.16 – 0.36	7.68 – 8.88	+
7	Dunnerholme	282 – 9200	0.20 – 0.97	6.79 – 7.57	+
8	Sandside	3020	1.6	6.72	+
9	Sharp Street	303 – 473	0.10 – 0.16	7.04 – 7.62	+
10	Subberthwaite	144.3	0.20	7.05	-
11	Bowness	950 +	0.13	7.13 – 7.74	+
12	Grune	90 – 1200	0.33 – 1.67	7.56 – 9.94	+
13	Mawbray	68.1 – 1350	0.13 – 0.49	7.42 – 8.25	+
14	Anthorn	734 – 6640	0.07 – 0.36	7.09 – 7.32	-
15	Ainsdale	402	0.15	7.22	+
16	Frensham	303	0.10	7.01	-
17	Sandy	405	0.29	7.37	+

Table 4.4. Salinity and pH measurements across all *E.calamita* sites (“Red” = Saline sites, “Blue = Freshwater sites, “Green” = Saline and Fresh sites)

4.3 *B.d.* rt-PCR results

A binomial two-tailed test was used to compare the prevalence of positive against negative samples across all 17 sites. This identified a significantly negative prevalence at a 95% confidence level ($p < 0.05$, p -value = 0.0002). The same statistical test was used to determine the prevalence of IPC positives against negatives. There was no significant association identified across the 6 sample sites ($p > 0.05$, p -value = 0.6875). There was also no significant association in the prevalence of positives identified when BSA ($p > 0.05$, p -value = 0.08) and Environmental TaqMan ($p > 0.05$, p -value = 0.08) were used on the 6 sample sites.

A binomial two proportion test was used to compare the prevalence levels of *B.d.* between saline and freshwater ponds. A highly significant difference was observed ($df = 1$, $p > 0.05$, p -value = 1.19310^{-08}) in the prevalence of positives between saline and freshwater ponds. A

two sample t-test was used to compare the genomic equivalents of GE scores and no significant association was determined ($p > 0.05$, p -value = 0.3175).

A Generalised Linear Model (GLM) Logistic Regression with Binomial Errors analysis was used to measure the temporal variation amongst sites 11-14 (Table 4.2), from the years 2009 – 2011. A significant variation was detected between the prevalence of infection over the four years ($df = 2$, $p < 0.001$, p -value = 0.00116, Figure 4.2). Also Table 4.2. illustrates that sites 11 and 14 are consistently negative or low over the three years, in contrast to sites 12 and 13 which are consistently high.

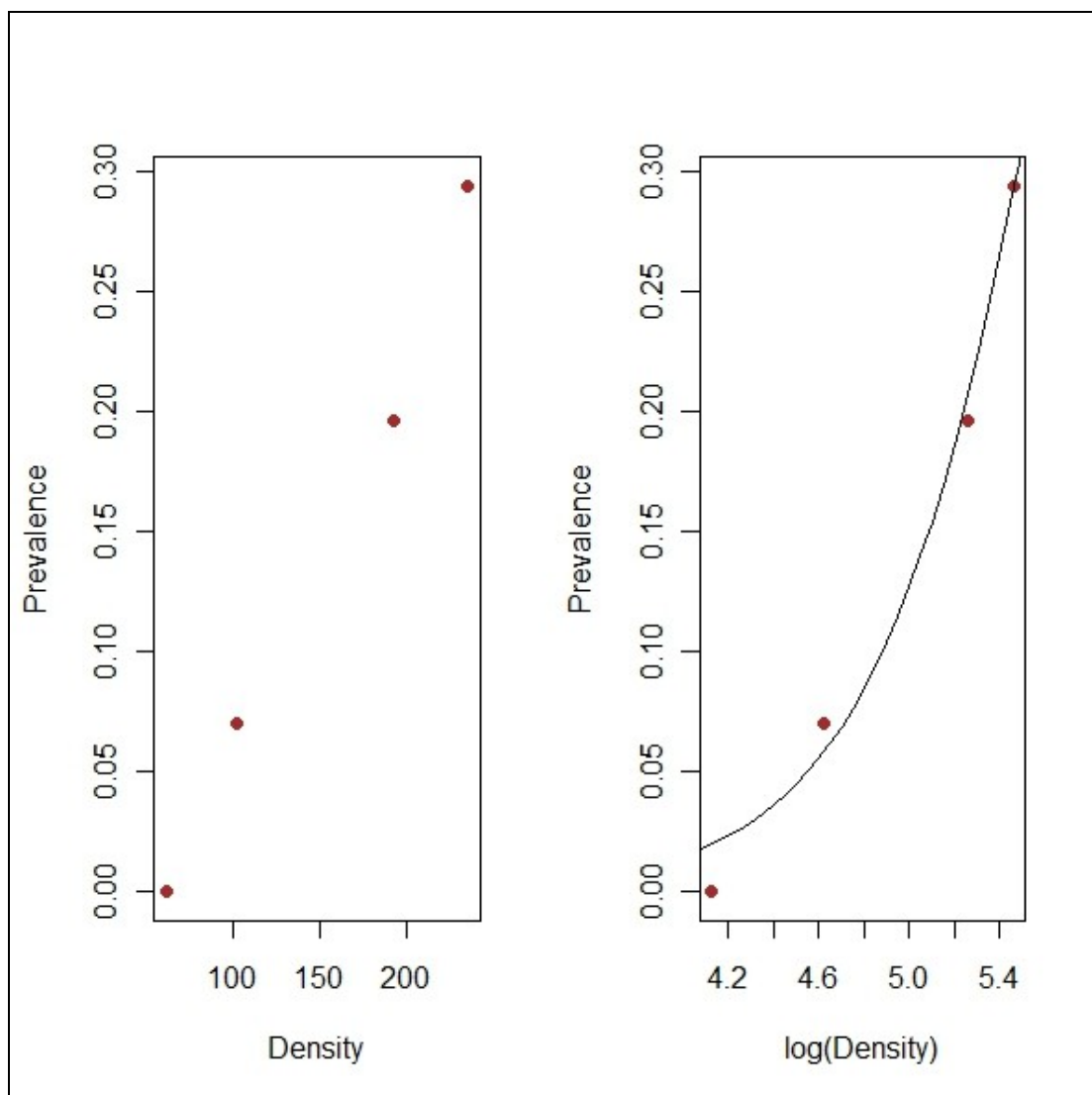


Figure 4.2. Temporal variation in *B.d.* prevalence at 4 *E.calamita* sites ("Density" = Total sample size)

*See Table 4.2 for site references/names

4.4 Effects of salinity and pH on *B.d.* rt-PCR results

The effects of salinity and pH were analysed against the *B.d.* infection results presented in Tables 4.1 and 4.3.

A Generalised Linear Model (GLM) Logistic Regression with Binomial Errors analysis was carried out to measure the impacts of salinity variance on the prevalence levels of *B.d.* at *E.calamita* sites. No significant association was detected when analysing prevalence against the titration measurements ($df = 38$, $p > 0.05$, $p\text{-value} = 0.507$). However a highly significant relationship was determined when analysing prevalence against the electrical conductivity recordings, as measures of salinity ($df = 38$, $p < 0.001$, $p\text{-value} = 0.00517$). This indicates that there may be a correlation between *B.d.* scores and salinity.

Using the family = binomial, the initial residual deviance was badly over dispersed (135.47 on 38 df) with an over-dispersion factor of 3.565. Therefore the model was re-fitted using family = quasibinomial to account for this variation.

The results are illustrated in Figure 4.3.

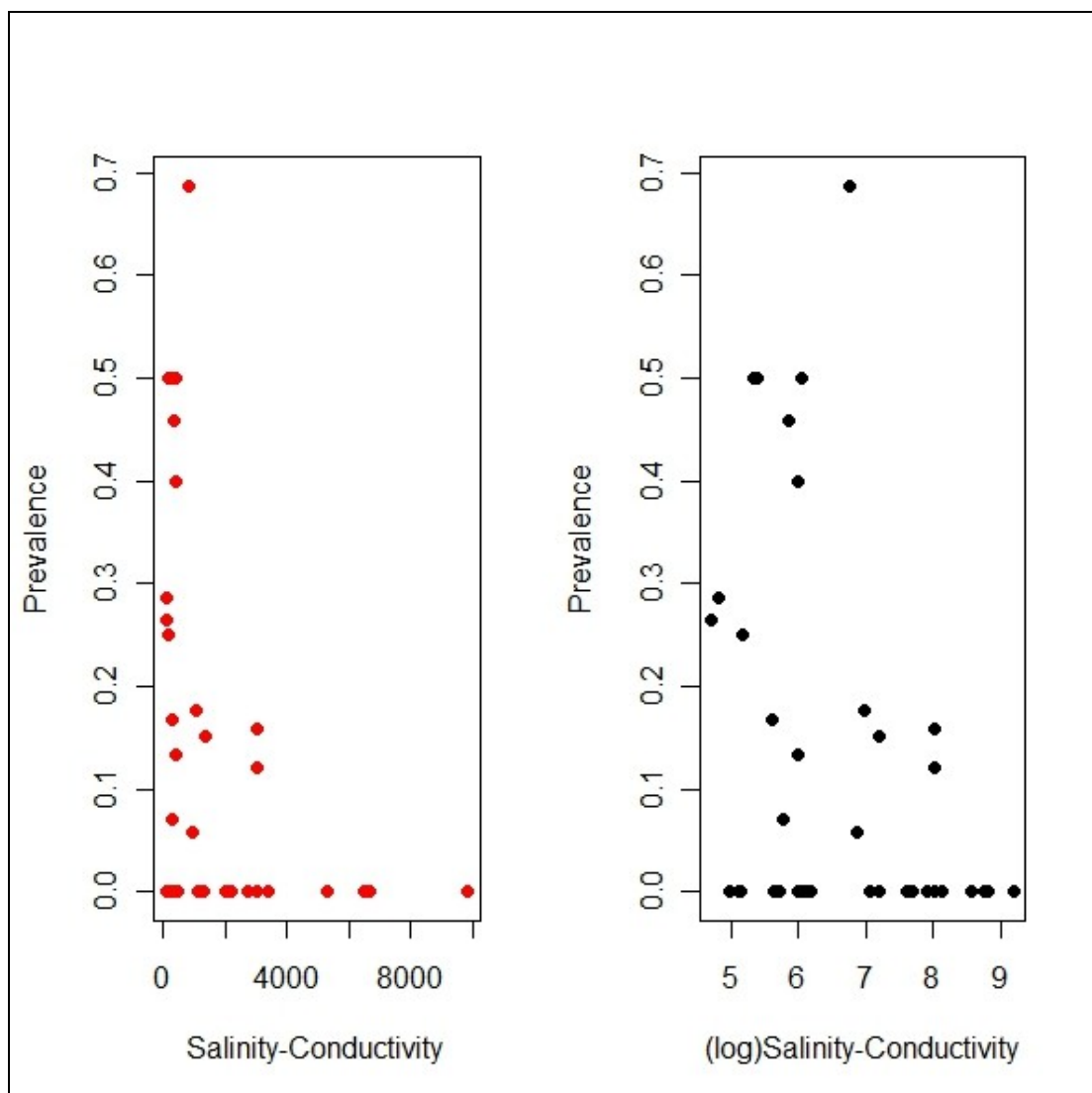


Figure 4.3. Effect of salinity on the prevalence of *B.d.* at *E.calamita* sites. (Family = quasibinomial)

A Generalised Linear Model (GLM) Logistic Regression with Binomial Errors analysis was carried out to measure the impacts of pH variance on the prevalence levels of *B.d* at *E.calamita* sites. A highly significant association was detected against the pH measurements (df = 38, $p < 0.001$, p-value = 0.00234) indicating that there may be a strong correlation between pH measurements and *B.d.* scores. Figure 4.4 also seems to represent that between the pH levels of 7 and 8.5, *B.d.* scores are substantially higher.

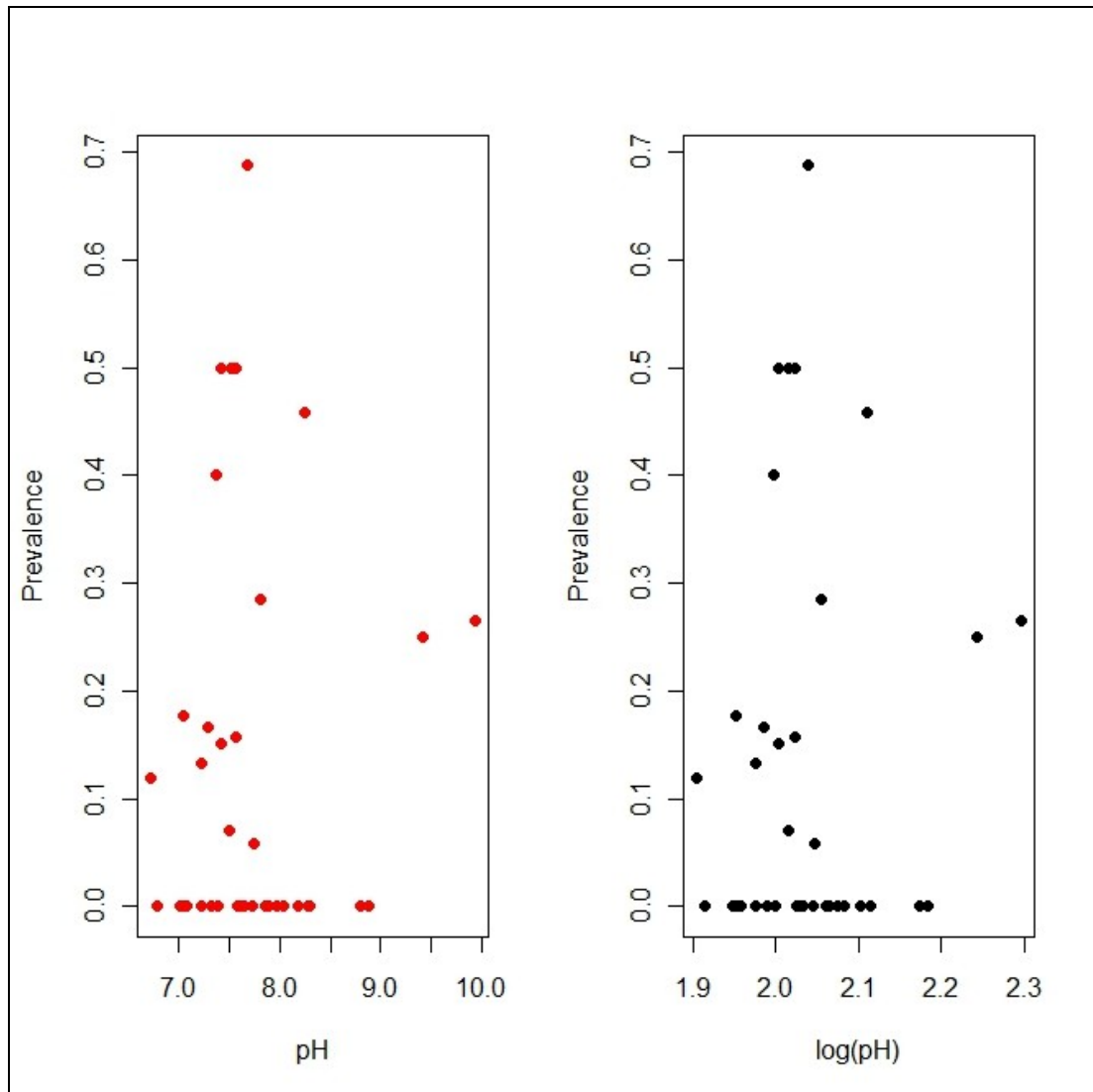


Figure 4.4. Effect of pH on the prevalence of *B.d.* at *E.calamita* sites. (Family = binomial)

Nevertheless when using a GLM model to analysis pH and conductivity as a measure of salinity together as continuous factors against the prevalence levels of *B.d.* no significant association presented ($p > 0.05$, p-value = 0.658). This suggests that pH and salinity are exerting strong impacts on an independent basis.

4.5 Synergy between salinity and pH

Electrical conductivity and chemical titration were both used as methods for measuring the level of salinity present. In order to determine whether there was a correlation between these measurements a logarithmic correlation coefficient analysis was used. The correlation

co-efficient (0.3403890, Figure 4.5) generated indicates there wasn't a significant association between the two measurements.

To account for this independence, the salinity measurements were analysed individually against the *B.d.* infection results.

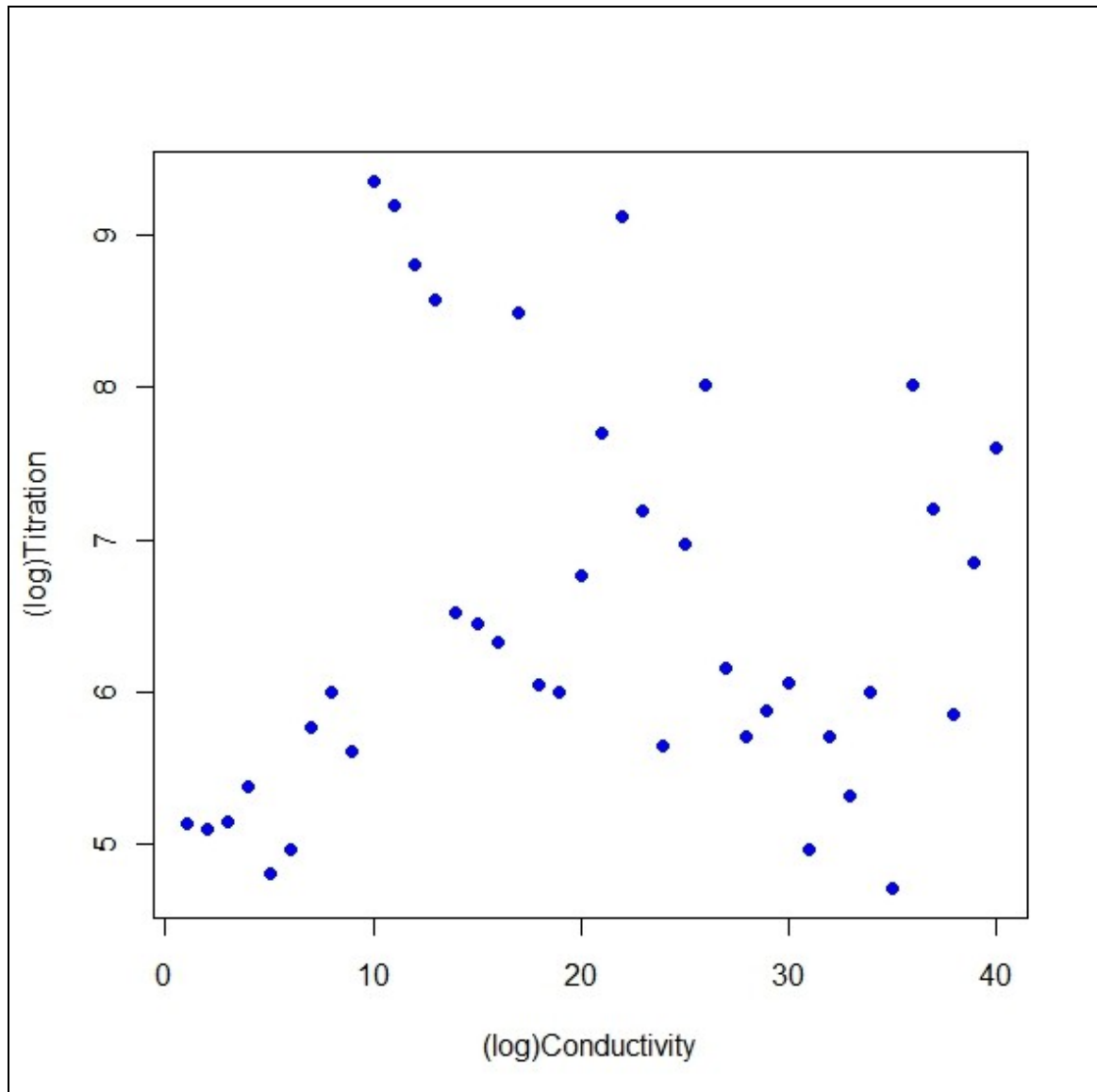


Figure 4.5. Correlation between two measurements of salinity (electrical conductivity, chemical titration)

The pH and salinity measurements were also analysed for any association using the logarithmic correlation coefficient. The correlation co-efficient (0.0197020, Figure 4.6) indicates that there wasn't a significant relationship between these two measurements.

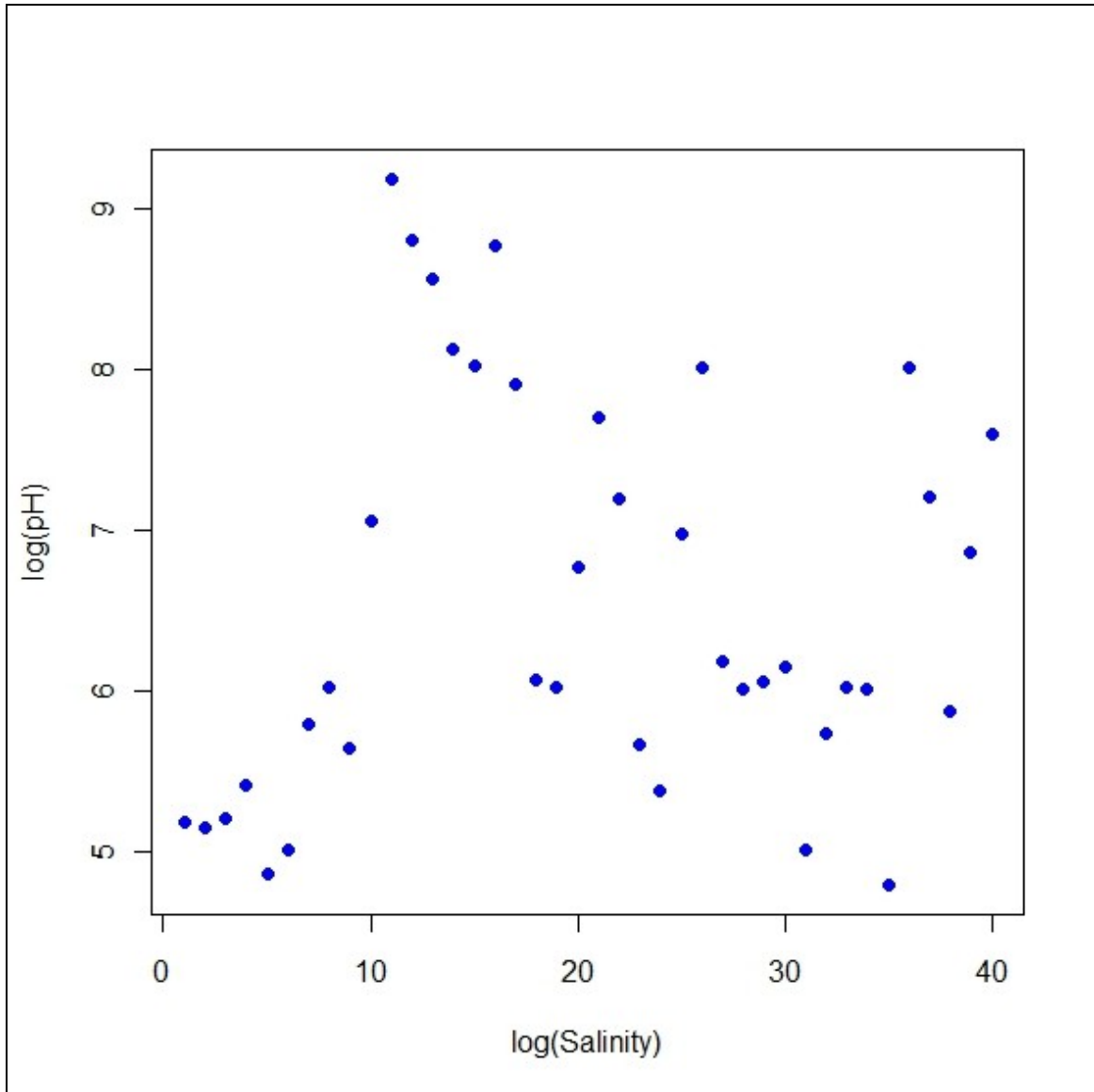


Figure 4.6. Correlation between salinity and pH as measurements of environmental co-factors.

4.6 Spatial patterns

A Generalised Linear Model (GLM) Logistic Regression with Binomial Errors was used to analyse any regional patterns across the Cumbrian sites. The sites located outside Cumbria were not incorporated into this analysis, due to a small sample size. No significant association was determined between the latitudes of the Cumbrian sites, and the prevalence levels of *B.d.* ($p > 0.05$, $p\text{-value} = 0.600$, Figure 4.7). Appendix III contains the geographical coordinates used for each Cumbrian site.

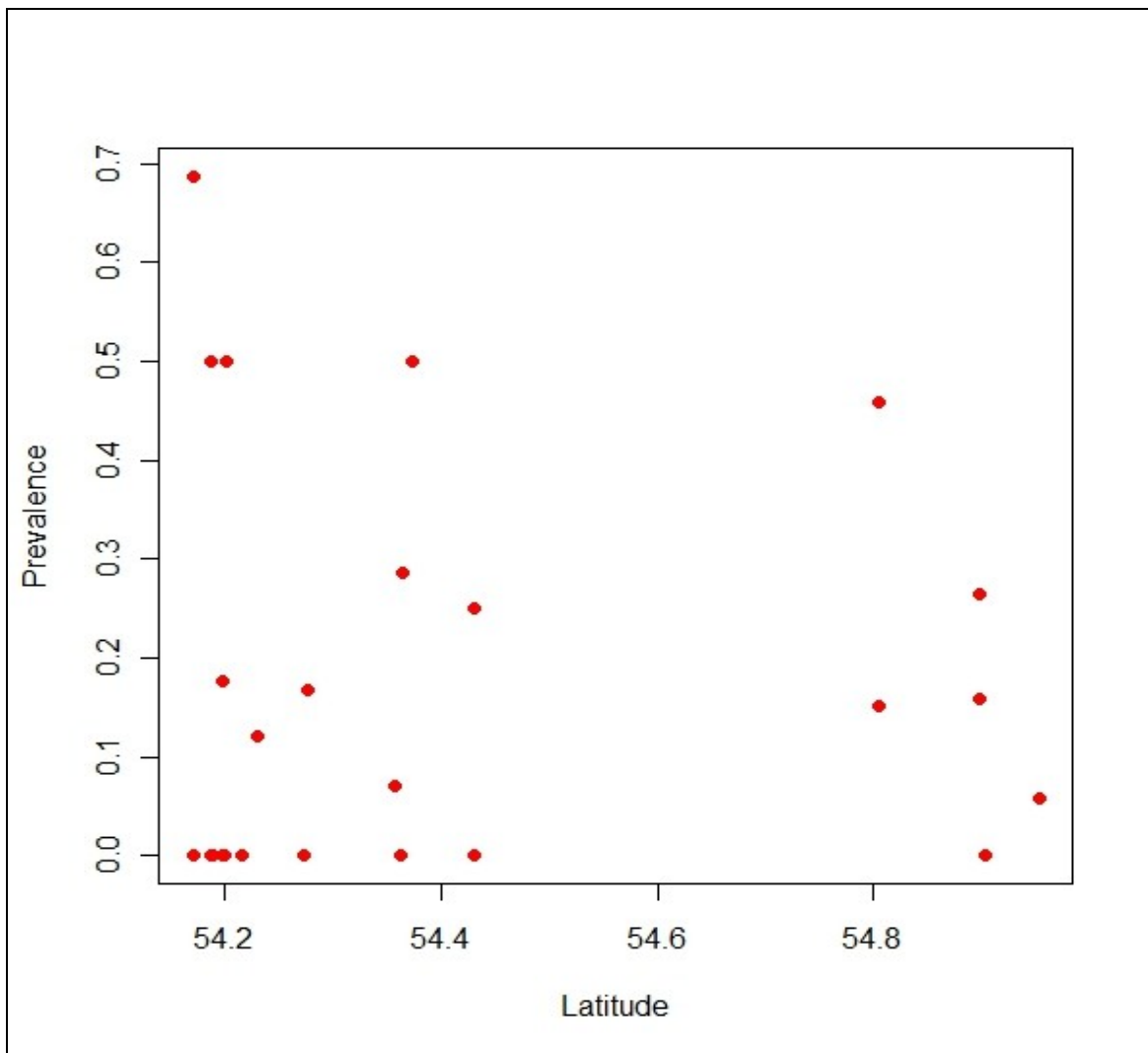
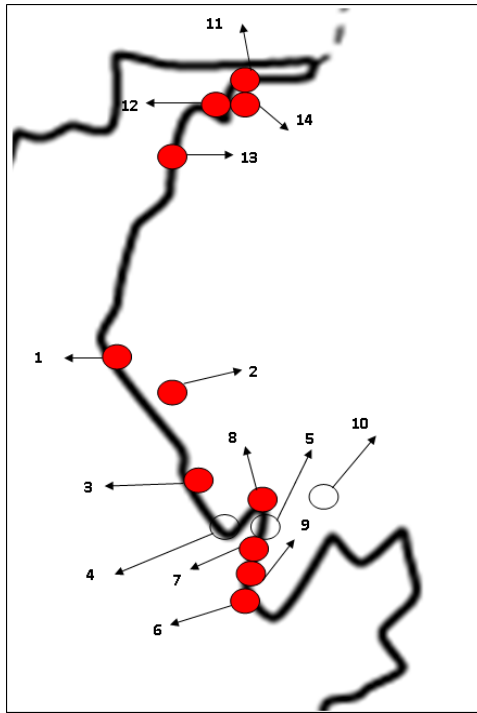


Figure 4.7. Spatial comparison between "Latitude" and "Prevalence" across 14 Cumbrian sites

4.7 Inhibition rt-PCR results

Internal Positive Controls (IPCs) were used to test for any inhibitory effects on the samples which may have been particularly susceptible, such as those from high saline environments.

Initially 6 sites (2, 4, 7, 8, 9 and 10, Table 4.1.) were analysed using IPC. Three of these were high saline sites, and the remainder were fresh.

IPC inhibition was identified at all three of the saline sites (4, 7 and 8), and within two of the fresh locations (2 and 10).

Bovine Serum Albumin was then used across the same samples to determine whether it neutralised any of the inhibitory effects. Both sites 4 and 10 had one sample that was identified as entirely inhibited, which subsequently turned positive when BSA was used. Furthermore at sites 2, 7, and 8 low rt-PCR repeats, presented as true positives (amplification in both wells).

The rt-PCR, IPC and BSA results across the 6 sites are presented in Table 4.5.

Site Number	Location	rt-PCR results (+/-) *	IPC Inhibition (+/-)	BSA (+/-)
2	Drigg	5/30	1/18	5/18
4	Haverigg	0/26	24/26	1/26
7	Dunnerholme	4/30	7/14	7/14
8	Sandside	3/25	3/23	3/23
9	Sharp Street	2/8	0/3	2/3
10	Subberthwaite	0/1	1/1	1/1

Table 4.5. IPC inhibition and BSA results across 6 Cumbrian sites ("Orange" = Inhibited *B.d.* sites)

* rt-PCR results refer to the standard results in Table 4.1. for all of the samples at each site.

Environmental TaqMan was also used as an alternative reagent across all the samples. Site 2 had one sample that was identified as entirely inhibited which turned positive when Environmental TaqMan was used. Furthermore at sites 2 and 4 some of the samples which were initially entirely inhibited subsequently presented as low rt-PCR repeats.

The rt-PCR, IPC and Environmental TaqMan results across the 6 sites are presented in Table 4.6.

Site Number	Location	rt-PCR results (+/-) *	IPC Inhibition (+/-)	Env TaqMan(+/-)
2	Drigg	5/30	1/18	2/18
4	Haverigg	0/26	24/26	1/26
7	Dunnerholme	4/30	7/14	6/14
8	Sandside	3/25	3/23	0/23
9	Sharp Street	2/8	0/3	1/3
10	Subberthwaite	0/1	1/1	1/1

Table 4.6. IPC inhibition and Env TaqMan results across 6 Cumbrian sites ("Orange" = Inhibited *B.d.* sites)

* rt-PCR results refer to the standard results in Table 4.1. for all of the samples at each site.

4.8 Effects of salinity on rt-PCR inhibition

As a result of the widespread inhibition identified in our samples within Section 4.7, all of the 13 sites were re-run and analysed for *B.d.* prevalence with and without Environmental TaqMan. The rt-PCR results from all 13 sites are displayed in Table 4.7.

Site Number	Location	Without Environmental TaqMan		With Environmental TaqMan	
		Proportion	Low Repeats *	Proportion	Low Repeats *
1	Braystones	1/8	1/8	1/8	2/8
2	Drigg	5/30	15/30	3/30	10/30
3	Annaside	1/6	1/6	2/6	1/6
4	Haverigg	0/26	0/26	1/26	4/26
5	Soutergate	0/5	0/5	1/5	2/5
6	Sandscale	21/42	6/42	23/42	5/42
7	Dunnerholme	4/30	2/30	13/30	5/30
8	Sandside	3/25	4/25	1/25	5/25
9	Sharp Street	2/8	0/8	4/8	1/8
10	Subberthwaite	0/1	0/1	1/1	0/1
15	Ainsdale	4/30	5/30	2/30	4/30
16	Frensham	0/2	0/2	0/2	1/2
17	Sandy	2/5	0/5	1/5	2/5

Table 4.7. rt-PCR results of 13 sites before and after the addition of Environmental TaqMan

* Low PCR results which have only amplified within one well (In addition to true positives where amplification occurs in both wells)

A Generalised Linear Model (GLM) Logistic Regression Model with Binomial Errors was carried out to measure for any difference between the prevalence of *B.d.* before and after Environmental TaqMan was used. Initially the rt-PCR low repeats were excluded from the analysis, to determine whether any significant difference was detected between the true positives. A significant association was observed without (df = 11, p < 0.001, p-value = 0.00165) and with the rt-PCR low repeats (df = 11, < 0.001, p-value = 0.000184). This indicates that Environmental TaqMan can reveal positive *B.d.* results which have been hidden by inhibition and generate an increase in estimated *B.d.* prevalence.

Evidently it also appears that there is an increase in the number of rt-PCR low repeats where *B.d.* has only been detected in one amplification well.

A paired T-test was carried out to compare the genomic equivalent (GE) scores of each positive sample before and after treatment with Environmental TaqMan. There was no significant association between the two samples (df = 118, p-value = 0.07605) which suggests that Environmental TaqMan did not reveal *B.d.* scores to be significantly higher once inhibition had been removed, in contrast to the finding of a slight increase in prevalence. Appendix I contains details of the genomic equivalent (GE) score for each rt-PCR positive before and after the addition of Environmental TaqMan.

Three predominantly saline sites (4, 5 and 7) appear to have marginally more positives when Environmental TaqMan is included; however this pattern isn't presented throughout as

other sites do not appear to exhibit the same pattern (2, 6 and 8). No remarkable differences are present between the samples within the freshwater sites.

5. Discussion

5.1. Environmental variability affects *B.d.*

Alterations in abiotic factors are predicted to have a dynamic effect on host-pathogen relationships and the complex life cycles of free living waterborne infectious diseases like *B.d.* These alterations will ultimately have an impact on the presence and prevalence of infection, and will expose the physiological limitations of disease.

pH and salinity were measured as key environmental factors of disease, which are subject to seasonal variation, alterations in tidal inundation, and changes in alkalinity and acidity. They have the ability to influence the infectivity and multiplication ability of disease dynamics which will ultimately have an impact on the persistence of infectious diseases in aquatic regions.

Gleason *et al* (2006) suggests that chytrids are susceptible to climatic variation and unable to grow in saline estuaries, instead preferring environments with low osmotic potentials. Similar research by Chukanhom & Hatai (2004) and White (2006) have found that freshwater and soil chytrids spores react negatively and often are inactivated after prolonged exposure to high levels of salinity above 5%. Piotrowski *et al* (2004) and Stallknecht *et al* (1990) have suggested that pH had a marked effect on the infectivity of chytridiomycosis in areas with a higher acidic content (< pH 6.0).

Interestingly, the results of this investigation indicated that the effects of pH and salinity had a highly statistically significant effect on the variability of *B.d.* within the environment. Salinity had a highly significant association when analysed as a continuous variable on the presence of *B.d.* as did pH. However when pH and salinity were analysed as joint variables to determine whether there was any interactive effect, there was no significant association. This suggests that pH and salinity are exhibiting important effects on the dynamics of chytridiomycosis independently from each other. Therefore it is essential that they are considered separately when investigating disease dynamics and planning future management strategies.

There was no significant association between titration and conductivity as measurements of salinity. The lack of correlation between the two salinity measurements may suggest that one of these didn't work efficiently; possibly titration, as these results didn't correspond as well as conductivity with field observations of ponds that were subject to tidal inundation (Minting, pers. comm). As a consequence the conductivity measurement was used for all "salinity" analyses.

The likelihood of changes in abiotic levels which undoubtedly will impact on the host-pathogen relationship is particularly significant as climate change becomes more of a pressing issue. Salinity and pH are likely to be particularly susceptible to climatic variations

due to increases in temperature and changes in aquatic composition. Furthermore often salt and pH levels are not static and will change in accordance with rain, sea spray and, evaporation.

Blaustein *et al* (2010) suggest that climate change will alter amphibian habitats dramatically, effecting vegetation, soil and hydrology which will influence host-pathogenic relationships and competitive interactions which may alter community structure. Bosch *et al* (2007) also suggested that there is substantial support for the chytrid thermal optimum hypothesis as a driver of chytridiomycosis.

Undoubtedly climatic variation, which will impact on pH and salinity, will also depend on the influence of other factors such as amphibian community composition, host density and microbial competitors.

Nevertheless the importance of understanding future trends and parameters is vital for future research on climatic sensitive diseases such as chytridiomycosis. Models that allow the definition of critical parameters and offer a means of developing more sophisticated methods for prediction of disease outbreaks will become imperative. Especially if we are to fully understand the impacts a changing environment will have on factors such as pH and salinity which are clearly crucial in managing the prevalence of *B.d.* at amphibian sites. Lipp *et al* (2002) have used a similar dynamic model to determine the association of factors such as water temperature and salinity on the transmission of cholera as climatic variations occur.

5.2 pH and salinity affect the prevalence and presence of *B.d.*

B.d. was shown to be directly correlated with the levels of pH and salinity, as demonstrated by their significant associations with the *B.d.* scores. This provides a strong indication that the presence of *B.d.* is highly influenced by the levels of pH and salinity present within the environment.

There was a significant association between the proportional data variance in sites 11, 12, 13, and 14 between 2009 and 2011 (Table 4.2). This suggests that the prevalence of *B.d.* may be locally affected by environmental factors which persist across years. However, these data were collected as part of an alternative research program and, no consistent pH and salinity measurements were recorded, so we are unable to test this hypothesis.

There is a significant association between the prevalence of *B.d.* across saline and freshwater sites. However it is important to note that there was an uneven split between saline and freshwater sites. For instance, the total surveyed natterjack sites included eleven freshwater sites, compared to only 6 saline sites. Also the ability to conduct a within-site comparison was hindered as only sites 6, 7 and 12 had sufficient sample sizes across freshwater and saline ponds.

There was no significant association between the genomic equivalent (GE) scores within sites which had saline and freshwater ponds. Nevertheless despite there being no statistical relationship, on a few occasions' high GE scores presented at freshwater sites 1, 2, and 15. Furthermore at site 2 one sample when analysed with IPC and Environmental TaqMan rose from a GE score of 0.0482 to 689.241. This is a large increase and suggests inhibition can occasionally mask high scores. Additionally at site 7, the GE scores of two samples from saline sites, rose when IPC and Environmental TaqMan were used. However it has been difficult to disentangle the effects of PCR inhibition and salinity upon *B.d.* scores, so therefore more research is needed to be certain that observed patterns are due to an effect of salinity upon chytridiomycosis.

These results appear to indicate that pH and salinity are exhibiting a higher impact on the presence and the probability of infection of *B.d.*, rather than on the GE scores which provide insight into *B.d.* growth rates and infectivity. The inability to identify any significant differences in GE scores between saline and freshwater sites could be due to the limited sample sizes at several of the sites. Although the samples from site 7 appear to suggest that salt is maintaining GE scores at lower levels, more research is needed to conclusively identify the cause of the inhibition.

In light of the significant impacts pH and salinity are exhibiting on the ability of *B.d.* to survive and multiply within these environments, it is crucial that environmental factors are incorporated into the future management of natterjack populations. Future habitat management strategies could be adapted to allow tidal inundation into a proportion of breeding ponds, and limit the prevalence of *B.d.* within natterjack sites.

White (2006) suggested pre-treating water used in amphibian ponds, and then later de-salinising it so that salt-sensitive species are not affected. However realistically this would only be feasible in captivity and regularly monitored permanent ponds. Additionally it is not presently known, how long a toad would need to be exposed, to be effectively disinfected. Denton *et al* (1997) suggested creating a network of ponds of varying depths and with varying levels of pH and salinity. Furthermore Banks & Beebee (1993) suggested using a number of mechanisms to manually control the pH and salinity levels of ponds. These included pre-treating water, adding lime into the water and excavating the peat substrate, both of which result in pH elevation. However the creation of adequate ponds requires a delicate balance and often involves substantial commitment. The location strategies applied when establishing new ponds could be vital in providing an environment which minimises the presence of *B.d.*, in the long-term at comparatively low cost.

A further consideration when designing suitable management infrastructure will be to ensure salinity levels are at an optimum level for natterjack toads to prevent any degradation of their natural physiological and breeding mechanisms.

Beebee (1985) suggested that total salinity can rise to lethal levels following tidal surges into coastal pools and marshes, which may have a detrimental effect on the reproductive ability of natterjack toads. Furthermore growth rates of tadpoles were inhibited by pH 4.0 – 6.0, with total mortality of spawn occurring below pH 4.0. White (2006) observed the effects of salt tolerance on Bell frogs and suggested that tadpoles and adult frogs do have some tolerance of salinity. Tadpoles were found to be able to tolerate salinity of up to 5% sea water (i.e. 1.7ppt) before there were any apparent effects on growth and maturation rates. Adult frogs however were able to tolerate 8% sea water for longer durations.

An experimental study in Cumbria which observed natterjack toad's reaction to saline and freshwater environments found that those kept within a saline environment fared significantly better (Minting, pers. comm). Toads kept in 1% salinity gained on average 2 grams per toad (33g in total) in comparison to the freshwater toads which lost 0.9 grams per toad. This may be suggestive that a certain level of salinity is required to maintain the electrolyte balance at equilibrium. This could be particularly telling as chytridiomycosis has been hypothesised to cause damage to skin function which results in disturbance to water or electrolyte balance (Berger *et al*, 2005).

5.3 *B.d.* is highly distributed across coastal sites

There was a significant negative prevalence across the *E.calamita* sites surveyed, with low rt-PCR positives included and without.

However *B.d.* appears to be widespread across the surveyed sites, with 12 out of 17 sites testing positive to some degree for *B.d.*

5.4 Spatial analysis of *B.d.* patterns

There was no significant pattern between the latitudes of the Cumbrian sites and the prevalence levels of *B.d.* detected. Sites located outside Cumbria were not included in this analysis due to the small number of sites. The lack of a statistical finding between latitude and *B.d.* prevalence appears to suggest that *B.d.* is widespread and similarly prevalent at natterjack sites in Northern, Western and Southern Cumbria.

Research by Arai (2008) examined spatial autocorrelation across the United Kingdom including within Cumbria and also found no spatial autocorrelation amongst sites with different disease statuses (positive and negative), indicating that no obvious clustering of positive or negative sites was present.

5.5 Environmental variability affects the detection ability of rt-PCR

The advancement of molecular technologies have led to the development of real-time TaqMan assays, however the full potential of rt-PCR has often been hindered by components in biological samples and organic detritus. The presence of salt has also been suggested as a potential inhibitor (Davalieva & Efremov, 2010).

Initially sites 2, 4, 7, 8, 9 and 10 (Table 4.1) were tested for inhibition using Internal Positive Controls (IPC). IPC inhibition was determined as partial at 40ct or above, and total when no amplification of either well occurred, or at 50ct and above.

On average the Ct value across all 6 sites was 38.56, however there was a marked difference between the first plate which included saline sites 4 and 7 and had an average Ct value of 48.30, and plate 2 which contained a mixture of saline and fresh sites (2, 8, 9 and 10) and had a Ct value of 35.13. Haverigg (4) experienced the most inhibition with 24 out of the 26 samples either partially or totally inhibited. The remaining saline sites (7, 8) were also inhibited to some degree. Fresh water sites also had samples which were identified as inhibited, but fewer samples were affected. This may suggest that the samples were contaminated with organic detritus and/or mud collected from the toad's skin. Only the freshwater site 9 suffered no inhibition in any of the samples.

Due to this marked inhibition across the 6 sites two reagents were used independently for their ability to neutralise inhibition (Kreader, 1996; Plante *et al*, 2010).

Bovine Serum Albumin (BSA) had no significant effect on reducing the inhibition in samples. Although both sites 4 and 10 had one sample each which presented as completely inhibited (> 50ct) and subsequently turned positive once BSA was used. Also at sites 2, 7 and 8 low rt-PCR repeats, presented as true positives with the addition of BSA.

Environmental TaqMan also had no statistically significant effect on reducing the inhibition in samples. Nevertheless all of the Ct values reduced to in-between the normal range of 35 – 37 Ct. Site 2 had one sample which was entirely inhibited (>50ct) and turned positive once Environmental TaqMan was used. Furthermore at sites 2 and 4 several samples which had previously been completely inhibited (>50ct) subsequently presented as low rt-PCR repeats.

As a result of the inhibition identified, all of the samples were re-run with Internal Positive Controls and Environmental TaqMan. There was a significant association observed between the prevalence of *B.d.* before and after Environmental TaqMan was used with the low rt-PCR repeats included and without. This suggests that Environmental TaqMan had a significant impact on reducing the inhibition present in the samples, although we are unable to define whether this was a result of salt or due to general organic detritus and/or mud.

No significant association was detected between the GE scores of each positive sample before and after the addition of Environmental TaqMan. This suggests that Environmental TaqMan is not having a significant effect on the level of genomic DNA within the samples.

Saline sites 4, 5 and 7 appear to have marginally more positives when Environmental TaqMan is included, however this pattern isn't significant and isn't replicated throughout, as sites 2, 6 and 8 do not exhibit the same pattern. No significant differences are identifiable between the samples from the freshwater sites.

5.6 Critical examination of the limitations and biases

A number of factors should be considered when interpreting the results from the data collected within this study.

The number of individuals swabbed at some of the sites was small, which may have limited the ability to detect infection. For example, 9 out of the 17 sites had a sample size of fewer than thirty individuals. With these numbers a prevalence level below 15% is unlikely to be detected at a 99% confidence level (DiGiacomo & Koepsell, 1986). Therefore although no positive animals were initially detected at sites 4, 5, 10, 14 and 16 there is only a limited level of confidence when suggesting that they are truly negative. Also at sites 1, 2, 6, 7 and 9 secondary visits were made to the ponds to try and maximise the sample size, therefore there is a possibility that some of the samples may not be independent from each other.

The number of individuals present at the sites was often dictated by the environmental conditions. Whilst in Cumbria, we experienced two dry hot spells which caused a large number of the non-permanent ponds to dry up. This resulted in the death of any spawn, and meant that the ponds were temporarily unsuitable as natterjack breeding sites. This hindered the sampling efforts, as even sites with permanent ponds experienced limited or no natterjack activity during this period.

Although all individuals were rinsed with freshwater prior to swabbing to prevent any inhibition from organic detritus and salt, the Internal Positive Controls revealed a large amount of inhibition across saline and freshwater sites. This suggests that all samples should be run with a reagent that limits inhibition to account for this factor. In addition although reagents like BSA and Environmental TaqMan which were used within this study limit inhibition we were unable to quantify the element that is causing this suppression of sensitivity.

The ability to detect any temporal trends in the effects of pH and salinity on the prevalence and presence of *B.d.* was hindered by the lack of long-term data. Although 11, 12, 13 and 14 have been surveyed as part of an alternative study for the last three years, no consistent pH and salinity data had been collected.

Spatial analysis was limited by the small sampling size (14 locations). Therefore, it was not clear whether the non-significant result was obtained due to the small sample size or due to no actual spatial clustering in the distribution of *B.d.*

5.7 Recommendations

5.7.1 Future Research

A similar study should be repeated in the locations sampled and others within different seasons and across different years. Repeating the study at sites which are susceptible to tidal inundation with high salinity ponds may be particularly beneficial. Longer temporal studies will help to identify variance in pH and salinity levels. They will also provide us with

invaluable information on the long-term effects that pH and salinity are having on the prevalence and presence of *B.d.*

pH and salinity levels should be recorded at all other *E.calamita* sites in the UK to determine whether the findings identified here are consistent at other natterjack sites, when *B.d.* is present. This will also increase the statistical power of the analysis and so may enable more statistically significant results to be identified.

All swab samples should be analysed with the inclusion of Internal Positive Controls. This will ensure that any inhibition is identified, and that the true target negatives are distinguished from PCR inhibition. If inhibition is occurring, all samples should be re-run with a reagent such as BSA or Environmental TaqMan that will limit these effects, and increase the sensitivity of the rt-PCR.

All other *E.calamita* sites in the UK must be surveyed for *B.d.* to ascertain their status in regard to *B.d.* distribution/infection in *E.calamita* populations. This will increase the statistical power of the analysis and provide us with a greater number of sites to perform a more robust spatial analysis.

5.7.2 Management Implications

Pond breeding amphibian species are known to experience natural population fluctuations (Marsh & Trenham, 2001), therefore to be able to detect the real trends in a population long-term monitoring across different seasons and years is required.

Climatic change is likely to affect the present composition of amphibian breeding sites and lead to changes in salinity and pH levels (Blaustein *et al*, 2010). Therefore to be able to identify temporal variance long-term monitoring is required on a larger scale. As new breeding ponds and scrapes are established the local strategies should be considered. Additionally the maintenance work should consider the topographical structure of the area. Ponds could be designed so that they receive regular inundation from tidal influx, and structured so that they provide semi-permanent, optimum breeding habitat for natterjack toads.

Where sites are already infected it is difficult to determine the best way to treat these ponds. A number of treatment methods in captivity are already available (White, 2006) where water is pre-treated with salt, however captive treatment is not a financially viable option for wild amphibian populations. We are also currently unsure as to the level of salt required to disinfect an amphibian, how long they need to be exposed for, and the tolerance levels of different species to salinity, in order to prevent any adverse effects.

5.8 Concluding remarks

This project aimed to investigate the apparent pattern of salinity and pH as environmental site factors that influence the prevalence and presence of *B.d.* Based on the results identified these environmental co-factors are having a highly significant impact on the

presence of *B.d.* within natterjack sites. Although these methods require further investigation, it is imperative to establish long-term trends on the effects of pH and salinity on *B.d.* so that local strategies can be designed and implemented by conservation managers and preventative management mechanisms established.

A second objective was to investigate the prevalence of *B.d.* at the surveyed sites through North West and Southern England. Although no significant positive correlation was identified, it is clear that *B.d.* is widespread and highly prevalent at a large number of the natterjack sites sampled. Although the spread of the disease was not covered within this study, previous research has identified that it is unlikely to remain localised due to its virulent and adaptable nature. Therefore it would be useful to establish long-term monitoring schemes, as well as determining the current status of *B.d.* infection at all *E.calamita* sites.

This project also investigated the effect of *B.d.* on a detection method after apparent inhibition was noticed in our samples. Although we were unable to determine the factor that was hindering the full potential of rt-PCR it is clear that despite the various precautionary measures followed rt-PCR was affected by contaminants within our samples. This highlights the importance of incorporating Internal Positive Controls into all rt-PCR surveillance work within the future. The advantages of other alternative methods should also be considered; an option for future work.

Chytridiomycosis may present a threat to the survival of natterjack toad populations in the United Kingdom. Nevertheless we have found that environmental co-factors have the ability to exert selective pressures on the host-pathogen relationship and suppress the prevalence and presence of infection. This study has focused on two environmental factors in particular, and found that even at a small-scale level they are exhibiting a significant impact on the intensity of infection. This project has also highlighted the need and opportunity to expand and broaden this specific research area. Further research could provide a more conclusive and detailed picture of the long-term impacts these environmental factors are exerting on this infection, and how management strategies could be adapted to help vulnerable amphibians in the future.

REFERENCES

- Al-Soud, W. A. & Radstrom, P. (1998) Capacity of Nine Thermostable DNA Polymerases to mediate DNA Amplification in the Presence of PCR-Inhibiting Samples. *Applied and Environmental Microbiology*, 64 (10), 3748-3753.
- Amon, J. P. & Arthur, R. D. (1981) Nutritional Studies of a Marine *Phlyctochytrium* Sp. *Mycologia*, 73 (6), 1049-1055.
- Arai, S. (2008) Investigation on the spread of chytridiomycosis between UK natterjack toads and other inland amphibian populations within Cumbria. *MSc Conservation Science: Imperial College London*.
- ARG, 2010 [Online] Available at: <<http://www.arguk.org>> [Accessed August 2011]
- Banks, B. & Beebee, T. J. C. (1993) Long-term management of a Natterjack Toad population in Southern Britain. *Biological Sciences*, 14 (2), 155-168.
- Beck, M. A. & Levander, O. A. (2000) Host nutritional status and its effect on a viral pathogen. *Journal of Infectious Diseases*, 182, S93-S96.
- Becker, C. G. & Zamudio, K. R. (2011) Tropical amphibian populations experience higher disease risk in natural habitats. *PNAS*.
- Beebee, T. J. C. (1985) Salt tolerances of natterjack toad (*Bufo calamita*) eggs and larvae from coastal and inland populations in Britain. *Herpetological Journal*, 1, 14-16.
- Beebee, T.J.C., Buckley, J. (2001) Natterjack Toad (*Bufo calamita*) Site Register for the UK 1970 – 1999 inclusive. Unpublished report by University of Sussex and The Herpetological Conservation Trust.
- Beebee, T. J. C., Flower, R. J., Stevenson, A. C., Patrick, S. T., Appleby, P. G., Fletcher, C., Marsh, C., Natkanski, J., Rippey, B. & Battarbee, R. W. (1990) Decline of the Natterjack Toad in Britain: Palaeoecological, documentary and experimental evidence for breeding site acidification. *Biological Conservation*, 53 (1), 1-20.
- Berger, L., Hyatt, A., Speare, R. & Longcore, J. E. (2005) Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 68, 51-63.
- Bielby, J., Cooper, N., Cunningham, A.A., Garner, T.W.J., Purvis, A. (2008) Predicting susceptibility to future declines in the world's frogs. *Conservation Letters*, 82-90.
- Bielby, J., Cunningham, A. A. & Purvis, A. (2006) Taxonomic selectivity in amphibians: ignorance, geography or biology? *Animal Conservation*, 9, 135-143.
- Blaustein, A. R. & Kiesecker, J. M. (2002) Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters*, 5, 597-608.

- Blaustein, A. R., Romansic, J. M., Kiesecker, J. M. & Hatch, A. C. (2003) Ultraviolet radiation, toxic chemicals and amphibian population declines. *Diversity and Distributions*, 97, 123-140.
- Blaustein, A. R., Walls, S. C., Bancroft, B. A., Lawler, J. J., Searle, C. L. & Gervasi, S. S. (2010) Direct and Indirect Effects of Climatic Change on Amphibian Populations. *Diversity*, 2, 281-313.
- Boots, M. (2011) The Evolution of Resistance to a Parasite is Determined by Resources. *The American Naturalist*, 178 (2), 214-220.
- Bosch, J., Carrascal, L. M., Duran, L., Walker, S. & Fisher, M. C. (2007) Climate change and outbreaks of chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society*, 274, 253-260.
- Bosch, J., Martinez-Solano, I. & Garcia-Paris, M. (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. *Biological Conservation*, 97, 331-337.
- Boyle, D. G., Boyle, D. B., Olsen, V., Morgan, J. A. T. & Hyatt, A. D. (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. *Diseases of Aquatic Organisms*, 60, 141-148.
- Briggs, C. J., Knapp, R. A. & Vredenburg, V. T. (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. *PNAS*, 107 (21), 9695-9700.
- Brown, J. D., Goekjian, G., Poulson, R., Valeika, S. & Stallknecht, D. E. (2009) Avian influenza virus in water: Infectivity is dependent on pH, salinity and temperature. *Veterinary Microbiology*, 136, 20-26.
- Buckley, J. & Beebee, T. J. C. (2004) Monitoring the conservation status of an endangered amphibian: the natterjack toad *Bufo calamita* in Britain. *Animal Conservation*, 7 (3), 221-228.
- Carey, C. & Alexander, M. A. (2003) Climate change and amphibian declines: is there a link? *Diversity and Distributions*, 9 (2), 111-121.
- Chukanhom, K. & Hatai, K. (2004) Freshwater fungi isolated from eggs of the common carp (*Cyprinus carpio*) in Thailand. *Mycoscience*, 45, 42-48.
- Collins, J. P., Brunner, J. L., Jancovich, J. K. & Schock, D. M. (2004) A model host-pathogen system for studying infectious disease dynamics in amphibians: Tiger Salamanders (*Ambystoma Tigrinum*) and *Ambystoma Tigrinum* virus. *Herpetological Journal*, 14, 195-200.
- Collins, J. P. & Storfer, A. (2003) Global amphibian declines: sorting the hypotheses. *Diversity and Distributions*, 9, 89-98.
- Crawley, M.J. (2005) *Statistics, An Introduction using R*. Chichester: John Wiley & Sons Ltd
- Cunningham, A. A., Garner, T. W. J., Aguilar-Sanchez, V., Banks, B., Foster, J., Sainsbury, A. W., Perkins, M., Walker, S. F., Hyatt, A. D. & Fisher, M. C. (2005) Emergence of amphibian chytridiomycosis in Britain. *Veterinary Record*, 157, 386-387.

- Daszak, P., Berger, L., Cunningham, A. A., Hyatt, A. D., Green, D. E. & Speare, R. (1999) Emerging Infectious Diseases and Amphibian Population Declines. *Emerging Infectious Diseases*, 5 (6), 735-748.
- Davalieva, K. & Efremov, G. D. (2010) Influence of salts and PCR inhibitors on the amplification capacity of three thermostable DNA polymerases. *Macedonia Journal of Chemistry and Chemical Engineering*, 29 (1), 57-62.
- Denton, J. S., Hitchings, S. P., Beebee, T. J. C. & Gent, A. (1997) A recovery program for the Natterjack Toad in Britain. *Conservation Biology*, 11 (6), 1329-1338.
- DiGiacomo, R. F. & Koepsell, T. D. (1986) Sampling for detection of infection or disease in animal populations. *American Veterinary Medical Association*, 189, 22-23.
- Ells, T. V., Stanton, J., Strieby, A., Daszak, P., Hyatt, A. D. & Brown, C. (2003) Use of Immunohistochemistry to Diagnose Chytridiomycosis in Dyeing Poison Dart Frogs (*Dendrobates tinctorius*). *Journal of Wildlife Diseases*, 39 (3), 742-745.
- Ferguson, C. M., Coote, B. G., Ashbolt, N. J. & Stevenson, I. M. (1996) Relationships between Indicators, Pathogens and Water Quality in an Estuarine System. *Water Research*, 30 (9), 2045-2054.
- Fisher, M. C. & Garner, T. W. J. (2007) The relationship between the emergence of *Batrachochytrium dendrobatidis*, the international trade in amphibians and introduced amphibian species. *Fungal Biology Reviews*, 21, 2-9.
- Fisher, M. C., Garner, T. W. J. & Walker, S. (2009) Global Emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in Space, Time and Host. *Annual Review of Microbiology*, 63, 291-310.
- Garner, T. W. J., Walker, S., Bosch, J., Leech, S., Rowcliffe, M., Cunningham, A. A. & Fisher, M. C. (2009) Life history tradeoffs influence mortality associated with the amphibian pathogen *Batrachochytrium dendrobatidis*. *Oikos*.
- Gleason, F. H., Daynes, C. N. & McGee, P. A. (2010) Some zoosporic fungi can grow and survive within a wide pH range. *Fungal Ecology*, 3, 31-37.
- Gleason, F. H., Kagami, M., Lefevre, E. & Sime-Ngando, T. (2008) The ecology of chytrids in aquatic ecosystems: roles in food web dynamics. *Fungal Biology Reviews*, 22, 17-25.
- Gleason, F. H., Midgley, D. J., Letcher, P. M. & McGee, P. A. (2006) Can soil Chytridiomycota survive and grow in different osmotic potentials? *Mycological Research*, 110, 869-875.
- Gleason, F. H., Schmidt, S. K. & Marano, A. V. (2010) Can zoosporic true fungi grow or survive in extreme or stressful environments? *Extremophiles*, 14, 417-425.
- Heuch, P. A., Knutsen, J. A., Knutsen, H. & Schram, T. A. (2002) Salinity and temperature effects on sea lice over-wintering on sea trout (*Salmo trutta*) in coastal areas of the Skagerrak. *Journal of the Marine Biological Association of the United Kingdom*, 82, 887-892.

- Hitchings, S. & Beebee, T. J. C. (1996) Persistence of British Natterjack Toad *Bufo calamita* Laurenti (*Anura: Bufonidae*) populations despite low genetic diversity. *Biological Journal of the Linnean Society*, 57, 69-80.
- Houlahan, J. E., Findlay, S., Schmidt, B. R., Meyer, A. H. & Kuzmin, S. L. (2000) Quantitative evidence for global amphibian population declines. *Nature*, 404, 752-755.
- Johnson, M. L., Berger, L., Philips, L. & Speare, R. (2003) Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. *Diseases of Aquatic Organisms*, 57, 255-260.
- Kiesecker, J. M. & Blaustein, A. R. (1995) Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. *Proceedings of the National Academy of Sciences USA*, 92, 11049-11052.
- Kilpatrick, A. M., Briggs, C. J. & Daszak, P. (2009) The ecology and impact of chytridiomycosis: an emerging disease of amphibians. *Trends in Ecology and Evolution*, 1-10.
- Koprivnikar, J., Baker, R. L. & Forbes, M. R. (2006) Environmental factors influencing trematode prevalence in grey tree frog (*Hyla versicolor*) tadpoles in Southern Ontario. *Journal of Parasitology*, 92 (5), 997-1001.
- Koprivnikar, J., Lim, D., Fu, C. & Brack, S. H. M. (2010) Effects of temperature, salinity and pH on the survival and activity of marine cercariae. *Parasitology Research*, 106, 1167-1177.
- Kreader, C. A. (1996) Relief of Amplification Inhibition in PCR with Bovine Serum Albumin or T4 Gene 32 Protein. *Applied and Environmental Microbiology*, 62 (3), 1102-1106.
- Kruger, K. M. & Hero, J. M. (2006) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. *Journal of Zoology*, 271 (3), 352-359.
- Lafferty, K. D. & Gerber, L. R. (2002) Good Medicine for Conservation Biology: the Intersection of Epidemiology and Conservation Theory. *Conservation Biology*, 16 (3), 593-604.
- Lei, F. & Poulin, R. (2011) Effects of salinity on multiplication and transmission of an intertidal trematode parasite. *Marine Biology*, 158, 995-1003.
- Lipp, E. K., Huq, A. & Colwell, R. R. (2002) Effects of Global Climate on Infectious Disease: The Cholera Model. *Clinical Microbiology Reviews*, 15 (4), 757-770.
- Lipp, E. K., Kurz, R., Vincent, R., Rodriguez-Palacios, C., Farrah, S. R. & Rose, J. B. (2001) The Effects of Seasonal Variability and Weather on Microbial Fecal Pollution and Enteric Pathogens in a Subtropical Estuary. *Estuaries*, 24 (2), 266-276.
- Lips, K. (1998) Decline of a Tropical Montane Amphibian Fauna. *Conservation Biology*, 12 (1), 106-117.
- Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. & Collins, J. P. (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *PNAS*, 103 (9), 3165-3170.

- Marsh, D.M. & Trenham, P.C. (2001) Metapopulation dynamics and amphibian conservation. *Conservation Biology*, 15(1), 40-49.
- Martin, L. B., Hopkins, W. A., Mydlarz, L. D. & Rohr, J. R. (2010) The effects of anthropogenic global changes on immune functions and disease resistance. *Annals of the New York Academy of Sciences*, 1195, 129-148.
- McGrath, A. L. & Lorenzen, K. (2010) Management history and climate as key factors driving natterjack toad population trends in Britain. *Animal Conservation*, 1-12.
- Moller, H. (1978) The effects of salinity and temperature on the development and survival of fish parasites. *Journal of Fish Biology*, 12 (4), 311-323.
- Mostowj, R. & Engelstadter, J. (2011) The impact of environmental change on host-parasite coevolutionary dynamics. *Proceedings of the Royal Society*, 278, 2283-2292.
- Muller, D. G., Kupper, F. C. & Kupper, H. (1999) Infection experiments reveal broad host ranges of *Eurychasma dicksonii* (Oomycota) and *Chytridium polysiphoniae* (Chytridiomycota), two eukaryotic parasites in marine brown algae (Phaeophyceae). *Phycological Research*, 47, 217-223.
- Nyvall, P. & Pederson, M. (1999) *THALASSOCHYTRIUM GRACILARIOPSISIS* (CHYTRIDIOMYCOTA), GEN. ET SP. NOV., ENDOSYMBIOTIC IN *GRACILARIOPSIS SP.* (RHODOPHYCEAE). *Journal of Phycology*, 35, 176-185.
- Perrigault, M., Bugge, D. M. & Allam, B. (2010) Effect of environmental factors on survival and growth of quahog parasite unknown (QPX) *in-vitro*. *Journal of Invertebrate Pathology*, 104 (2), 83-89.
- Piotrowski, J. S., Annis, S. L. & Longcore, J. E. (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. *Mycologia*, 96 (1), 9-15.
- Plante, D., Belanger, G., Leblanc, D., Ward, P., Houde, A. & Trottier, Y. L. (2010) The use of bovine serum albumin to improve the RT-qPCR detection of foodborne viruses rinsed from vegetable surfaces. *Applied Microbiology*, 52 (3), 239-244.
- Pounds, A. (2001) Climate and amphibian declines. *Nature*, 410, 639-640.
- Pounds, A. J. & Crump, M. L. (1994) Amphibian Declines and Climate Disturbance: The Case of the Golden Toad and the Harlequin Frog. *Conservation Biology*, 8 (1), 72-85.
- Rachowicz, L. J., Hero, J. M., Alford, R. A., Taylor, J. W., Morgan, J. A. T., Vredenburg, V. T., Collins, J. P. & Briggs, C. J. (2005) The Novel and Endemic Pathogen Hypotheses: Competing Explanations for the Origin of Emerging Infectious Diseases of Wildlife. *Conservation Biology*, 19 (5), 1441-1448.
- Reading, C. J. (2007) Linking global warming to amphibian declines through its effects on female body condition and survivorship. *Oecologia*, 151, 125-131.
- Rohr, J. R. & Raffel, T. R. (2010) Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. *PNAS*, 107 (18), 8269-8274.

- Santaliestra, M. E., Fisher, M. C., Fernandez-Beaskoetxea, S., Fernandez-Beneitez, M. J. & Bosch, J. (2011) Ambient Ultraviolet B Radiation and Prevalence of Infection by *Batrachochytrium dendrobatidis* in Two Amphibian Species. *Conservation Biology*, 1-8.
- Stallknecht, D. E., Kearney, M. T., Shane, S. M. & Zwank, P. J. (1990) Effects of pH, Temperature, and Salinity on Persistence of Avian Influenza Viruses in Water. *Avian Diseases*, 34, 412-418.
- Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S. L., Fischman, D. L. & Waller, R. W. (2004) Status and Trends of Amphibian Declines and Extinctions Worldwide. *Science Express*, 10, 1-3.
- Studer, A., Thieltges, D. W. & Poulin, R. (2010) Parasites and global warming: net effects of temperature on an inter-tidal host-parasite system. *Marine Ecology Progress Series*, 415, 11-22.
- Wake, D. B. (1991) Declining amphibian populations. *Science*, 253, 860.
- Walker, S. F., Bosch, J., Gomez, V., Garner, T. W. J., Cunningham, A. A., Schmeller, D. S., Ninyerola, M., Henk, D. A., Ginestet, C., Arthur, C. P. & Fisher, M. C. (2010) Factors driving pathogenicity vs prevalence of amphibian panzootic chytridiomycosis in Iberia. *Ecology Letters*, 13, 372-382.
- Walker, S. F., Salas, M. B., Jenkins, D., Garner, T. W. J., Cunningham, A. A., Hyatt, A. D., Bosch, J. & Fisher, M. C. (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms*, 77, 105-112.
- Weldon, C., du Preez, L. H., Hyatt, A. D., Muller, R. & Speare, R. (2004) Origin of the Amphibian Chytrid Fungus. *Emerging Infectious Diseases*, 10 (12), 2100-2105.
- White, A. W. (2006) A trial using salt to protect Green and Golden Bell Frogs from Chytrid infection. *Herpetofauna*, 36 (2), 93-96.
- Wilson, I. G. (1997) Inhibition and facilitation of nucleic acid amplification. *Applied Environmental Microbiology*, 63 (10), 3741-3751.
- Wolinska, J. & King, K. C. (2009) Environment can alter selection in host-parasite interactions. *Trends in Parasitology*, 25 (5).
- Woodhams, D. C., Alford, R. A., Briggs, C. J., Johnson, M. & Rollins-Smith, L. A. (2008) Life-History Trade-Offs Influence Disease In Changing Climates: Strategies Of An Amphibian Pathogen. *Ecology*, 89 (6), 1627-1639.
- Wu, C. S. & Kam, Y. C. (2009) Effects of salinity on the survival, growth, development and metamorphosis of *Fejervarya limnocharis* tadpoles living in brackish water. *Zoological Science*, 26 (7), 476-482.

APPENDICES

APPENDIX I – Genomic equivalent (GE) scores for positive samples at *E.calamita* sites

Site Number	Location	Zoospore * (Before Environmental TaqMan)
1	Braystones	34.72
2	Drigg	0.0758
		0.0482
		0.00324
		0.0867
		33.67
3	Annaside	1.41
6	Sandscale	0.662
		0.521
		1.06
		0.532
		0.515
		12.39
		0.0897
		0.652
		1
		27.04
		0.0424
		9.59
		2.31

		0.911
		18.34
		0.158
		2.53
		1.36
		0.194
		0.128
		0.434
7	Dunnerholme	6.6742
		0.0559623
		0.224909
		0.0784919
8	Sandside	0.292
		0.0286
		0.317
9	Sharp Street	0.185
		0.145
15	Ainsdale	0.000864
		0.00304583
		373.215
		9.5908E-09
17	Sandy	0.00000742
		0.0000466

* Genomic equivalents (GE) of zoospore numbers calculated using standards.
(GE >0.1 was regarded as positive, extraction dilution 1:10)

Site Number	Location	Zoospore * (After Environmental TaqMan)
1	Braystones	239.14
2	Drigg	2.04
		689.241
		172.77
3	Annaside	0.205
		1.6
4	Haverigg	0.309477
5	Soutergate	0.227
6	Sandscale	0.724815
		0.133037
		1.62105
		0.355021
		0.0851481
		0.295073
		0.0634511
		0.118154
		2.72427
		0.0274062
		0.0920931
		0.180175
		6.09422
		3.3455
		0.48441
0.275669		
2.47424		

		0.0277713
		0.460993
		0.233913
		0.0803048
		0.0254147
		0.148254
7	Dunnerholme	1.1701
		31.0302
		118.072
		0.650159
		1.11024
		0.782991
		0.815
		0.111
		0.0770837
		0.0308991
		0.597117
		0.305755
		2.31078
8	Sandside	0.354
9	Sharp Street	0.36
		0.061
		0.222
		1.47
10	Subberthwaite	0.353
15	Ainsdale	425.067

		0.064122
17	Sandy	0.189

* Genomic equivalents (GE) of zoospore numbers calculated using standards.
(GE >0.1 was regarded as positive, extraction dilution 1:10)

APPENDIX II – Salinity and pH readings from *E.calamita* sites

Site Number	Location	Salinity (uS)	pH
1	Braystones	164.8 – 178	7.55
		165 – 170	8.18
		164 – 170	8.28
2	Drigg	218	7.42
		122	7.81
		143	7.32
		405 – 4420	7.73
		321	7.50
3	Annaside	318	7.30
4	Haverigg	1101 – 11610	8.03
		1404 – 9830	8.29
		1222 – 6670	8.03
		1221 – 5280	7.97
		684 – 3400	7.88
		635 – 3050	7.86
		562 – 6480	7.64
5	Soutergate	2730 – 48600	7.08
6	Sandscale	300 – 426	8.79
		300 – 404	8.88
		863 – 980	7.68
7	Dunnerholme	1326	7.38
		282	7.22

		209	7.57
		433 – 1068	7.04
8	Sandside	30200	6.72
9	Sharp Street	473 – 482	7.04
		303 – 400	7.58
		357 – 421	7.51
		427 – 460	7.62
10	Subberthwaite	144.3	7.05
15	Ainsdale	402	7.22
16	Frensham	303	7.01
17	Sandy	405	7.37

APPENDIX III – Geographical coordinates of Cumbrian *E.calamita* sites.

Site Number	Location	Latitude	Longitude
1	Braystones	54.4308370709°	-3.5298661065°
		54.4309169490°	-3.5299461664°
		54.4309062082°	-3.5300845154°
2	Drigg	54.3733502037°	-3.4660707083°
		54.3645971206°	-3.4495976954°
		54.3619945697°	-3.4455198648°
		54.3620924383°	-3.4425990868°
		54.3579873397°	-3.4431477178°
3	Annaside	54.2776115652°	-3.4131664253°
4	Haverigg	54.1888280088°	-3.3088461597°
		54.1888875791°	-3.3083269615°
		54.1889200258°	-3.3078222313°
		54.1890005551°	-3.3070278251°
		54.1890531270°	-3.3063244900°
		54.1891103606°	-3.3060197755°
		54.1892251576°	-3.3053797024°
5	Soutergate	54.2160516404°	-3.1993196177°
6	Sandscale	54.1708370141°	-3.2257077642°
		54.1707349010°	-3.2260264442°
		54.1718824824°	-3.2245437645°
7	Dunnerholme	54.1993425126°	-3.2056725586°
		54.1993084120°	-3.2054876108°

		54.2017655556°	-3.2051452330°
		54.1984624926°	-3.2064900425°
8	Sandside	54.2302022043°	-3.1898963553°
9	Sharp Street	54.1872602926°	-3.2112823290°
		54.1873151293°	-3.2111919817°
		54.1872560834°	-3.2108071273°
		54.1873113818°	-3.2106708175°
10	Subberthwaite	54.2725800196°	-3.1444175087°

APPENDIX III – Examples of codes used for statistical analyses in R

- i) R script used, and the read-out obtained, for the generalised linear models, using salinity as a continuous variable as a function of *B.dendrobatidis* infection, as an example.

```
y <- cbind (Positive, Negative)
model <- glm (y ~ Salinity, family = quasibinomial)
summary (model)
```

Deviance Residuals:

Min	1Q	Median	3Q	Max
-4.2738	1.2933	-0.7894	-0.1322	5.6614

Coefficients:

	Estimate	Std. Error	t value	Pr (> t)
(Intercept)	-0.7280937	0.2017687	-3.609	0.000885 ***
Salinity	-0.0006311	0.0002127	-2.967	0.005177 **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Dispersion parameter for quasibinomial family taken to be 3.285217)

AIC: NA

Null deviance: 178.47 on 39 degrees of freedom

Residual deviance: 135.47 on 38 degrees of freedom

Number of Fisher Scoring iterations: 5