DISS. ETH Nr. 27188

Detection of aquatic wildlife pathogens from eDNA in water samples

Validation and Application in Switzerland

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zürich)

presented by

NATALIE MARIETTA SIEBER

Master of Science ETH in Environmental Sciences

born on 15.03.1991

citizen of Zurich, Switzerland

accepted on the recommendation of

Prof. Dr. Christoph Vorburger

Dr. Hanna Hartikainen

Dr. Armin Zenker

Prof. Dr. Jukka Jokela

Dr. Trude Vrålstad

2020

Table of Contents

Summary

Emergent diseases are contributing to the decline of various freshwater species already facing multiple threats of anthropogenic origin, such as habitat degradation, climate change and species introductions. One of the most ecologically important diseases is the crayfish plague, caused by the oomycete *Aphanomyces astaci*, responsible for widespread population collapses of native European freshwater crayfish species. The chytrid fungus *Batrachochytrium dendrobatidis* is another highly problematic pathogen and major contributor of amphibian declines worldwide, its spread being promoted by animal trade. *Saprolegnia parasitica*, closely related to *A. astaci* and causal agent of Saprolegniosis, widely occurs in freshwater habitats and can cause high mortality outbreaks in fish. Proliferative kidney disease (PKD), caused by the myxozoan *Tetracapsuloides bryosalmonae*, severely impacts wild salmonid stocks and trout farms. Due to its temperature-dependent development, climate change is expected to increase the disease's impact on host populations.

Mitigation and prevention of negative impacts caused by emergent diseases is of interest for species conservation and from an economic viewpoint. Therefore, effective management measures need to be devised, which in turn, require close and comprehensive surveillance of diseases and their agents. However, regularity and scope of monitoring campaigns are often impeded by the high costs and effort required for conventional disease monitoring methods, which often focus on the capture and examination of host species. Furthermore, such methods are usually limited to host species of the same taxonomic groups, such as fish or amphibians.

DNA acquired from the target organism's environment (e.g. water or soil) and not directly from the target itself, is called environmental DNA (eDNA). Using eDNA-based techniques, waterborne pathogens can be directly detected in water, omitting the need for laborious host capture, which renders them less cost- and time-intensive than conventional detection methods. Also, eDNA-based detection is more adaptable to detection of multiple pathogen species across taxonomic boundaries, since they are not limited to single host taxonomic groups. Therefore, eDNA-based methods could facilitate more regular and comprehensive disease monitoring campaigns.

The central aim of this thesis was to develop, test and apply an eDNA-based method and workflow for the detection of aquatic wildlife pathogens in water. More specifically, the four

species presented above, *A. astaci*, *B. dendrobatidis*, *S. parasitica* and *T. bryosalmonae* and their detection in water, using already published quantitative real-time PCR assays, was analysed.

In a controlled environment I tested the effect of water source and estimated zoospore concentrations on the reliability of *B. dendrobatidis* and *T. bryosalmonae* detection in water (Chapter 1). Detection consistency was surprisingly low for both pathogens, though even low estimated zoospore concentrations were detected. Likely reasons for the observed imperfect detection of the two pathogens could have been the heterogeneous distribution of zoospores in the water and variability in DNA extraction efficiency introduced by the filter.

The performance of the developed method for detecting *A. astaci* in water was compared to detection of the pathogen in crayfish tissue from individuals collected from the same sampling sites (Chapter 2). Results of the two methods only partly overlapped, indicating that reliable detection in asymptomatic carrier populations is challenging. A combination of both water and tissue sampling methods for surveillance could therefore create a more accurate picture of *A. astaci* occurrence.

In a country-wide survey, water samples were collected and analysed for the four pathogens *A. astaci, B. dendobatidis, S. parasitica* and *T. bryosalmonae*, applying the eDNA-based method I developed (Chapter 3). Widespread distribution of *A. astaci, S. parasitica* and *T. bryosalmonae* in water samples was found, reflecting prior surveys and expectations. Rare detection of *B. dendrobatidis* was likely due to inappropriate site selection for amphibians. This survey showed the feasibility of monitoring multiple pathogens using eDNA-based techniques, albeit limitations are still imposed by pathogen and host properties, such as habitat range.

In a concluding chapter, I discuss implications of the results of the previous chapters and propose ways for improvement. I further discuss areas of research and topics I deem important for the future development and application of eDNA-based methods for disease surveillance.

Zusammenfassung

Viele Süsswasserarten werden, nebst anthropogenen Beeinflussungen, wie Verschlechterung des Lebensraumes, Klimawandel und invasive Arteinführungen, durch neuaufkommende Krankheiten bedroht. Eine der gefährlichsten Krankheiten ist die Krebspest, welche vom Eipilz *Aphanomyces astaci* verursacht wird und den Kollaps vieler europäischer Flusskrebspopulationen herbeigeführt hat. Der Chytridpilz *Batrachochytrium dendrobatidis* ist ein weiterer problematischer Krankheitserreger, deren Verbreitung durch den internationalen Tierhandel begünstigt wird und mitverantwortlich ist für Rückgänge von Amphibienpopulationen weltweit. Die mit *A. astaci* nah verwandte *Saprolegnia parasitica*, Verursacherin von Saprolegniose, kommt in fast allen Süsswassergewässern vor und kann hohe Mortalitäten in Fischbeständen verursachen. Die Proliferative Nierenkrankheit (PKD), mit dem Myxozoten *Tetracapsuloides bryosalmonae* als Erreger, hat grosse negative Auswirkungen auf sowohl wilde Salmonide als auch in Fischzuchten. Durch ihre temperaturbedingte Entwicklung wird eine weitere Ausbreitung durch die Klimaerwärmung erwartet.

Die Eindämmung und Präventation von negativen Auswirkungen durch aquatische Krankheiten ist wichtig für den Artenschutz. Hierfür müssen effektive Massnahmen geplant und umgesetzt werden, was nur mit ausführlichem Wissen über das Vorkommen der Krankheitserreger möglich ist. Die Umsetzung von umfassenden Monitorings von solchen Krankheiten wird oft durch hohe Kosten und Aufwand erschwert. Konventionelle Methoden beinhalten meist das Einfangen von Wirtsindividuen und deren Untersuchung im Labor. Oft sind sie deswegen auch auf eine einzelne taxonomische Einheit von Wirtsarten beschränkt, z.B. Fische oder Amphibien.

DNA, welche der Umwelt des Zielorganismus (z.B. Wasser oder Boden) und nicht direkt dem Organismus, entzogen wird, nennt sich Umwelt-DNA (engl. eDNA). Auf eDNA basierende Methoden können Krankheitserreger direkt im Wasser nachweisen, ohne den Wirt suchen zu müssen und sind daher mit weniger Aufwand und Kosten verbunden als konventionelle Methoden. Zudem ist die Umwelt-DNA Methode flexibler beim Nachweis mehrerer Erregerarten aus verschiedenen taxonomischen Gruppen, weil sie weniger an eine Wirtsart gebunden ist. Umwelt-DNA Methoden könnten aus diesen Gründen die regelmässige und umfassende Überwachung von Krankheiten erleichtern.

Das Hauptziel dieser Arbeit war die Entwicklung, das Testen und Anwenden einer Umwelt-DNA Methode für den Nachweis von Krankheitserreger aquatischer Wildtiere in Wasser. Die vier erwähnten Erreger, *A. astaci*, *B. dendrobatidis*, *S. parasitica* und *T. bryosalmonae* und deren Nachweis in Wasser mithilfe bereits veröffentlichter quantitative real-time PCR Untersuchungen, wurden analysiert.

In kontrollierter Umgebung habe ich den Effekt von Wasserherkunft und geschätzter Zoosporenkonzentrationen auf die Nachweiszuverlässigkeit von *B. dendrobatidis* und *T. bryosalmonae* in Wasser getestet (Kapitel 1). Beide Erreger konnten nur unregelmässig in Wasserproben nachgewiesen werden. Dieses Muster könnte durch die ungleichmässige Verteilung der Sporen im Wasser und der Effizienzvariabilität der DNA Extraktion durch den Filter, verursacht werden.

Die in dieser Arbeit entwickelte Umwelt-DNA Methode wurde zudem mit einer konventionellen Methode für den Nachweis von *A. astaci* verglichen (Kapitel 2). Hierfür wurden sowohl Wasser- als auch Gewebeproben von Flusskrebsen am selben Standort analysiert. Die Resultate beider Methoden stimmten nur teilweise überein, was verdeutlicht, wie schwierig der verlässliche Nachweis des Erregers in asymptomatischen Wirtspopulationen ist. Eine Kombination beider Methoden für die Überwachung des Krankheitserregers könnte ein genaueres Bild der Verbreitung *von A. astaci* schaffen.

Die Verbreitung der vier Krankheitserreger *A. astaci*, *B. dendobatidis*, *S. parasitica* und *T. bryosalmonae* wurden in einer landesweiten Erhebung mittels Umwelt-DNA Methode untersucht (Kapitel 3). *A. astaci*, *S. parasitica* und *T. bryosalmonae* waren weit verbreitet, was frühere Untersuchungen und Erwartungen bestätigt hat. Der seltene Nachweis von *B. dendrobatidis* führt wahrscheinlich daher, dass die Wahl der Probestellen nicht typischen Amphibienhabitaten entsprach. Nichtsdestotrotz wird die Machbarkeit von Erhebungen zum Nachweis mehrerer Krankheitserreger mit Umwelt-DNA Methoden aufgezeigt, auch wenn gewisse Limitationen durch die Zielarten und deren Wirte gegeben sind.

Im abschliessenden Kapitel diskutiere ich über die Folgerungen aus der vorliegenden Arbeit und mögliche Wege zur Verbesserung der Methode. Zudem bespreche ich Gebiete und Themen, die ich für den Fortschritt und die Anwendung von Umwelt-DNA Methoden zum Nachweis von Krankheitserregern als wichtig wahrnehme.

General Introduction

Emerging diseases

Parasites and pathogens are ubiquitous in ecosystems and affect species interactions and coexistence (Freeland 1983, Price et al. 1988). However, many emerging diseases with serious detrimental effects on host populations have been registered in the last 50 years (Cunningham et al. 2017). Infectious diseases are defined as emerging if they recently increased their prevalence or geographic spread, after recent movement into new host populations or species, or if they are caused by newly evolved disease agents (Daszak et al. 2001). Human emerging infectious diseases (EID) frequently are of zoonotic origin, i.e. from animal disease agents that have evolved to infect humans (Cunningham et al. 2017). Global spread of HIV / AIDS, the Ebola virus outbreak in Africa, and, most recently, the coronavirus pandemic, are prominent examples of emerging zoonoses in humans. An increase in emerging diseases has also been observed in animals, which is of concern for the conservation of wild populations and for food safety and human health if domestic animals are affected (Daszak et al. 2000, Cunningham et al. 2017). For example, canine distemper virus has caused local extinctions in black-footed ferret (Thorne & Williams 1988) and population declines in many wild carnivore species, such as lions (Roelke-Parker et al. 1996). Emergent diseases have also been increasingly observed in freshwater ecosystems. They are thought to contribute to the decline of numerous aquatic species of conservation concern and they impact species important for aquaculture. For example, the nematode *Anguilliocoloides crassus* was introduced to Europe by importation of Asian eels (*Anguilla japonica*) and has spilled over to wild European eels (*Anguilla anguilla*; Koops & Hartmann 1989) and *Myxobolus cerebralis*, agent of whirling disease, has caused serious mortalities in wild salmonids after its emergence in North America (Hedrick et al. 1998).

Environmental change caused by anthropogenic activities is known to drive disease emergence in freshwater systems, most notably climate change (Marcogliese 2008, Karvonen et al. 2010, Okamura & Feist 2011) and species invasions (Stewart 1991, Peeler et al. 2011). The potential effects of climate change on host-pathogen dynamics are manyfold: increased water temperature can fasten parasite and host development differently, leading to changes in host-parasite interactions (Paull & Johnson 2011), it can increase virulence of pathogens (Cuco et al. 2018) and lead to ecosystem changes through elevated parasite-induced mortality of ecosystem engineer species (Larsen & Mouritsen 2014). Further, climate change can lead to range shifts, creating opportunities for novel host-parasite

combinations (Dunn & Hatcher 2015). Moreover, the frequent introduction of non-native species through global trade drives the establishment of invasive species and their parasites, which can have serious negative impacts on native species (see Peeler et al. 2011 for examples).

Challenges in disease management

Aquatic disease outbreaks have ecological, economic and human health-related consequences. The fishery and aquaculture industry has been growing rapidly since the 1950's and provides us with highly valuable products, therefore playing an important role in global food security (FAO 2020). Maintenance of healthy aquatic ecosystems is thus of great interest. Effective disease management strategies need to be devised for the mitigation and prevention of further negative impacts of diseases. This requires comprehensive knowledge of the prevalence and spread of diseases and their agents, which necessitates extensive and regularly updated monitoring. Disease management is currently more re-active than pro-active, in that action is frequently taken after negative impacts of a disease are noted, rather than being preventive of such impacts. For more pro-active management, close surveillance of diseases and their agents would be required to enable epidemiological predictions, pinpoint possible sources of invasions and identify regions most at risk. However, high costs and effort often impede the implementation of regular and comprehensive monitoring campaigns. Conventional disease monitoring usually encompasses the laborious capture and subsequent pathological examination of the host species, conducted by specialised personnel. Examination of a large number of host individuals is required for reliable results when disease agent prevalence is low in host populations, further increasing effort and cost (Schrimpf et al. 2013). Depending on the host and its disease, such methods can be detrimental to the host, and can even require their death (e.g. Wahli et al. 2002, Vrålstad et al. 2011). The development of a more cost-effective method for disease agent surveillance would therefore be beneficial for pro-active disease management.

Table 1. A non-exhaustive overview of pathogens of aquatic wildlife species for which eDNA-based methods for the detection in water have been developed. The table lists host species, the name of the disease, a short description on present research on detection in water and references for further reading.

Species	Host species	Disease	Short description	References
Aphanomyces astaci	freshwater crayfish	crayfish plague	Extensive research on the detection in water of the crayfish plague agent has been conducted. Simultaneous detection of crayfish host species has been applied and the detection in water was used for zoospore shedding experiments.	Strand et al. (2011, 2012, 2014, 2019b); Makkonen et al. (2013); Svoboda et al. (2013); Robinson et al. (2018); Wittwer et al. (2018a, 2018b, 2019); Rusch et al. (2020)
Batrachochytrium dendrobatidis	amphibians	chytridiomycosis	*B. dendrobatidis* detection in water has been subject to many studies and detection data has been frequently applied for occupancy modelling. A survey including citizen scientist for collecting eDNA samples for *B. dendrobatidis* analyis was conducted.	Kirshtein et al. (2007); Walker et al. (2007); Hyman & Collins (2012); Schmidt et al. (2013); Chestnut et al. (2014); Kamoroff & Goldberg (2017); Mosher et al. (2017, 2018); Julian et al. (2019); Barnes et al. (2020)
Ceratonova shasta	polychaete (*Manayunkia speciosa*), fish (Salmonidae)	enteronecrosis	*C. shasta* is an important disease agent of North American salmonids. Temporal dynamics in DNA concentrations in water were observed and could be correlated to host mortality. Further, *C. shasta* detection in water showed significant associations to host detection in water.	Hallett & Bartholomew (2006); Hallett et al. (2012); Richey et al. (2020)
Myxobolus cerebralis	oligochaete (*Tubifex tubifex*), fish (Salmonidae)	whirling disease	*M. cerebralis* was detected at low levels in water samples.	Richey et al. (2018)
Parvicapsula minibicornis	polychaete (*Manayunkia speciosa*), fish (Salmonidae)	not named	A qPCR assay was developed for *P. minibicorinis* and applied in a river system. Water sampling revealed the widespread distribution of the parasite within the river system and seasonal fluctuations in abundance.	Hallett & Bartholomew (2009)
Perkinsus marinus	oyster (*Crassostrea virginica*)	Perkinsiosis	A qPCR assay for the pathogen was developed and tested on water samples.	Audemard et al. (2004, 2006)

Table 1. (cont.)

Species	Host species	Disease	Short description	References
Ranavirus	ectothermic vertebrates	not named	Ranavirus detection in water has been measured following amphibian life cycle stages and was observed to correlate with later stages of tadpole development. Further, ranavirus abundance in water correlated with viral titres in larval tissues. Ranavirus analysis of water samples was included in the citizen science project mentioned above for *B. dendrobatidis*.	Robert et al. (2011); Hall et al. (2016, 2018); Julian et al. (2019); Miaud et al. (2019)
Saprolegnia parasitica	sapro- and necrotrophic pathogen	saprolegniosis	A qPCR assay for the detection of *S. parasitica* in water was developed after high mortality outbreaks in the river Loue. The pathogen was detected in the river water but not in tap water of nearby settlements.	Rocchi et al. (2017)
Tetracapsuloides bryosalmonae	bryozoans, fish	proliferative kidney disease	*T. bryosalmonae* detection in water was tested in rivers and coupled with detection of its bryozoan primary host. A metacommunity model of a river catchment was created including eDNA data from *T. bryosalmonae* and its bryozoan host.	Fontes et al. (2017); Carraro et al. (2017, 2018); Hutchins et al. (2018)

Environmental DNA for pathogen detection in water

The growing field of environmental DNA (eDNA) techniques and their application in conservation biology, biodiversity studies and invasion ecology (see Taberlet et al. 2012, and Thomsen & Willerslev 2015 for comprehensive reviews) offers opportunities also for pathogen surveillance. Contrary to direct sampling of DNA of a target organism, eDNA is acquired through sampling of the organism's environment, e.g. water, soil, air (Taberlet et al. 2012). One of the biggest advantages of eDNA sampling is the relative ease with which samples can be collected, since isolation of the target organism is not required. The idea of retrieving DNA from environmental samples has been investigated since the end of last century (Holm-Hansen et al. 1968), the term eDNA was first mentioned by Ogram et al. (1987) and began to be used frequently in the early 2000's, mostly by microbiologists (e.g. Henne et al. 1999, Rondon et al. 2000). Today, numerous studies have been published which have successfully applied eDNA methods for detection of a broad range of aquatic species, both targeting single species using species-specific assays, such as amphibians (Pilliod et al. 2013, Spear et al. 2015), crayfish (Tréguier et al. 2014, Mauvisseau et al. 2019) and fish (Turner et al. 2014, Laramie et al. 2015), and multiple species using sequencing and metabarcoding (e.g. Hänfling et al. 2016 for fish, Deiner et al. 2017 for a review). With a large list of species that have been investigated with eDNA techniques, efforts to develop standardised protocols and procedures (Goldberg et al. 2016, Jeunen et al. 2019, Thalinger et al. 2020 preprint) are important for further progress of the field.

Environmental DNA techniques can also be implemented for the detection in water of parasites and pathogens with waterborne life stages, though not as eDNA in the strict sense, i.e. extra-organismal DNA (Lacoursière-Roussel & Deiner 2019, but see Pawlowski et al. 2020 for a less restrictive definition), but rather as whole organisms, such as free-swimming zoospores or eggs. The concept of pathogen detection in water using molecular tools was investigated already in the early nineties (e.g. Toranzos et al. 1993, Arvanitidou et al. 1997) with a focus on human waterborne pathogens (Aw & Rose 2012). Opportunities for eukaryote parasites were also investigated (Bass et al. 2015) and novel molecular analytical methods are experiencing rapid advancements (Bonadonna et al. 2019). Water quality assessments by measuring bacterial and eukaryote pathogen abundance in water are now routinely used and incorporated in international directives (e.g. Schets et al. 2002). Molecular detection methods have been applied for many important human waterborne parasites and pathogens, such as *E. coli* (Ahmed et al. 2008), *Enterococcus* (Haugland et al. 2005), *Giardia*

lamblia and *Cryptosporidium parvum* (Guy et al. 2003), Aichi virus and human bocavirus (Shaheen et al. 2020), *Salmonella typhimurium* (Dupray et al. 1997), *Schistosoma mansoni* (Sato et al. 2018, Sengupta et al. 2019) and Vibrio sp. (Lipp et al. 2003, Rivera et al. 2003, Turner et al. 2014, Mok et al. 2019). An overview of aquatic wildlife parasites and pathogens investigated with eDNA-based detection techniques is found in Table 1. Besides monitoring disease agents for their abundance and spread in aquatic ecosystems, eDNA-based detection in water can be applied in live animal trade for health control of traded animals by sampling the water in which they are transported (Smith et al. 2012, Trujillo-González et al. 2019, Brunner 2020) Disease surveillance is especially important for animals to be released into local aquatic systems, such as live bait (Mahon et al. 2018) or for reintroduction efforts.

Direct detection in water using eDNA-based techniques removes the need to capture and examine affected hosts, significantly decreasing the effort and costs of disease monitoring. Furthermore, DNA extraction and processing are more easily adaptable and applicable to a wide range of target species compared to capture and examination, which require specialised knowledge depending on the host species. Molecular detection methods could therefore help establish monitoring schemes that provide comprehensive and up-to-date knowledge of disease occurrence for informed management decisions.

Study system

In this thesis, we developed, validated and applied an eDNA-based method for the detection of aquatic wildlife pathogens in water. The method was tested on four aquatic pathogens of concern presented in the following sections.

Aphanomyces astaci

The causative agent of the crayfish plague, one of the most serious aquatic wildlife diseases, is the oomycete *Aphanomyces astaci* (Order: Saprolegniales). In Europe, *A. astaci* poses a major threat for highly susceptible native freshwater crayfish species, contributing to their decline and local extinctions, while invasive North American crayfish species act as reservoir species (Holdich et al. 2009). Free-swimming *A. astaci* zoospores have two flagella, which are shed once the spore encysts on the host surface and becomes sticky. The cyst then germinates, and mycelia starts growing and spreading in the crayfish cuticle before re-emerging from the host to form sporangia containing primary cysts which are then released as zoospores (Svenson 1978, Cerenius and Söderhäll 1984 in Cerenius et al. 1988). The pathogen usually enters the crayfish through lesions in the epicuticle, the outermost layer of

the exoskeleton (Unestam & Weiss 1970). In the North American signal crayfish (*Pacifastacus leniusculus*), further growth of *A. astaci* hyphae is effectively stopped or slowed by a fast melanisation response, frequently rendering infections asymptomatic. Similar reactions were observed in European native noble crayfish *Astacus astacus* but developed too slowly to prevent fatal infection (Nyhlén & Unestam 1980, Cerenius et al. 2003). In late infection stages, *A. astaci* hyphae grow outwards from heavily infected parts of the exoskeleton (Unestam & Weiss 1970 ; Fig. 1).

A. astaci is suspected to have originally invaded Europe via crayfish transport in ballast water of trans-Atlantic ships (Holdich 2003) and the first outbreak was recorded in Italy in 1859, followed by many outbreaks throughout Europe (Alderman 1996). Stocking efforts using *P. leniusculus* with no known disease status, to counter dwindling native crayfish populations, further promoted the spread and exacerbated the problem posed by *A. astaci* (Bohman et al. 2006). Today, invasive North American crayfish and *A. astaci* are found throughout Europe (Kouba et al. 2014). The most widespread North American crayfish species in Europe, including Switzerland, are *P. lenisusculus*, the spiny-cheek crayfish (*Faxonius limosus*) and the red swamp crayfish (*Procambarus clarkii*). While *F. limosus* has mostly settled in larger waterways and *P. clarkii* prefers small lakes and ponds, *P. leniusculus* is spreading from main waterways into small streams, further displacing native populations, whose remaining refugia mostly are isolated waterbodies or small, remote streams (Stucki & Zaugg 2005). For effective implementation of management plans (Stucki & Zaugg 2011, Elmiger et al. 2018), close surveillance of native and invasive crayfish populations and *A. astaci* occurrence is crucial.

Batrachochytrium dendrobatidis

The chytrid fungus *Batrachochytrium dendrobatidis* (Order: Rhizophydiales) has become infamous as the agent of chytridiomycosis. This disease has caused mass mortalities in amphibians worldwide (Fisher et al. 2009), and is thought to be a major driver of extinction of frog species (Skerratt et al. 2016, Cunningham et al. 2017). *B. dendrobatidis* is a generalist pathogen of amphibians and infections vary in severity between species, from no symptoms (Daszak et al. 2004) to high mortalities (Lips et al. 2006). Free-swimming zoospores move short distances using their flagellum (Piotrowski et al. 2004) and encyst within 24 hours after their release from the zoosporangium (Berger et al. 2005). The process of infection remains unknown, but Longcore et al. (1999) suggest that after encysting, zoospores inject their content through a germ tube. In the skin, the spore develops into a thallus with a

zoosporangium. Mature zoosporangia contain fully formed zoospores which are then discharged into the water (Berger et al. 2005).

Chytridiomycosis was first observed by Berger et al. (1998) in anurans from Australia and Central America and *B. dendrobatidis* was described by Longcore et al. (1999). Several factors could contribute to the emergence of chytridiomycosis (Rachowicz et al. 2005): Vector species introductions from amphibian trade, such as the bullfrog (*Lithobates catesbeianus*; Daszak et al. 2004) facilitate and accelerate its spread and make new potentially highly susceptible populations accessible to the pathogen. Furthermore, *B. dendrobatidis* occurrence and severity has been associated to environmental factors such as temperature, precipitation and biome (Olson et al. 2013). Therefore, environmental change, together with species invasions, could shape *B. dendrobatidis* emergence patterns.

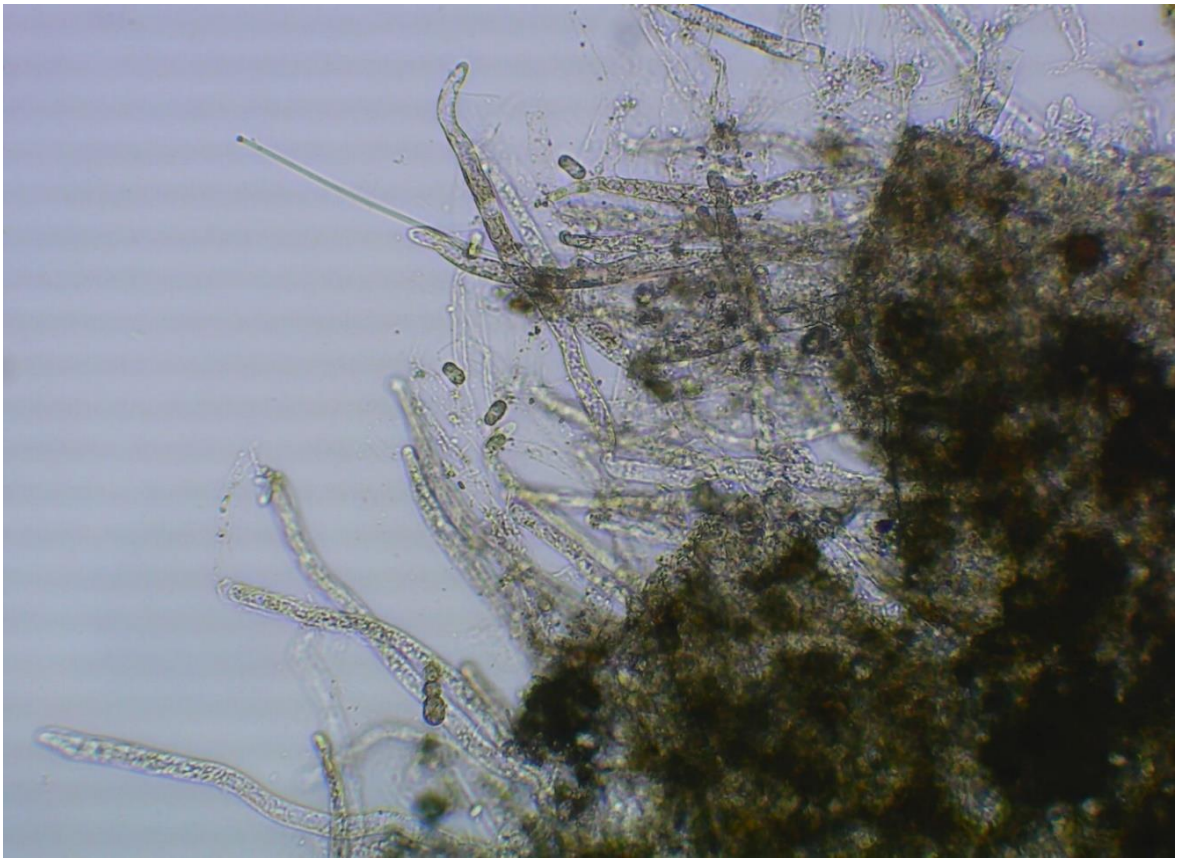


Figure 1. *Aphanomyces astaci* hyphae growing out of noble crayfish (*A. astacus*) tissue (Photo: N. Sieber)

Saprolegnia parasitica

Another oomycete closely related to *A. astaci*, *Saprolegnia parasitica* is endemic to all freshwater habitats and belongs to the commonly called "water moulds". While *S. parasitica* is generally considered an opportunistic sapro- and necrotrophic pathogen, some strains can

be highly virulent (Neish 1977) and cause high annual losses in aquaculture (van West 2006). High mortality outbreaks in fish caused by *S. parasitica* have been observed in the wild, such as in the French-Swiss border river Doubs (Paul & Belbahri 2012). *Saprolegnia parasitica* zoospores are biflagellate and can encyst several times before attaching to a suitable host (van West 2006). After first encystment, the zoospores develop long hooked hairs that are thought to help attachment to the host. Once *S. parasitica* has attached and invaded a host's epidermal tissues it develops and grows until forming sporangia at the end of hyphal cells that release new zoospores into the environment (Bruno & Wood 1999 van West 2006). Its widespread distribution in freshwater and its opportunistic nature pose a challenge for disease management, since its detection in a waterway does not necessarily indicate immediate disease risk.

Tetracapsuloides bryosalmonae

Proliferative kidney disease (PKD) causes great economic losses in salmonid fish farming and wild populations due to high mortalities in young-of-the-year salmonid fish (Clifton-Hadley et al. 1984, Hedrick et al. 1993). PKD is widespread in wild salmonid populations in North America (Hedrick et al. 1993, Kent et al. 1995) and Europe (El-Matbouli & Hoffmann 2002, Feist et al. 2002, Sterud et al. 2007, Wahli et al. 2007) and is suspected to play a major role in declines of affected fish species (Okamura et al. 2011), also in Switzerland (Burkhardt-Holm 2002, Borsuk et al. 2006). The disease is temperature driven (Bettge et al. 2009a, 2009b) and water eutrophication can promote its growth (Hartikainen et al. 2009). This implies that with increasing water temperatures due to climate change and further eutrophication, PKD outbreaks and their severity might increase in the future (Okamura et al. 2011).

First description of the disease dates back almost a century (Clifton-Hadley et al. 1984), while the disease agent was identified as a member of the myxozoa in 1985 by Kent and Hedrick (1985) and finally identified as *Tetracapsuloides bryosalmonae* by Canning et al. (1999). While the disease has been well-described in fish, freshwater bryozoans are the agent's primary hosts (Canning et al. 2000). *T. bryosalmonae* first develops a covert infection, i.e. the infection is not visible via stereomicroscopy, in its bryozoan host. During overt infections visible sacs containing spores are developed and spores are released into the environment. *T. bryosalmonae* infections in bryozoans can alternate between covert and overt stages. In water the spores remain viable for approximately 24 h (De Kinkelin et al. 2002) and infect fish through skin or gills (Morris et al. 2000, Longshaw et al. 2002). *T.*

bryosalmonae develops one to two months in the fish kidney. Spores released in urine by fish (Hedrick et al. 2004) are only infective to bryozoans, not to other fish (Morris & Adams 2006, Grabner & El-Matbouli 2008).

Current state of conventional pathogen surveillance in Switzerland

Conventional monitoring of all four pathogens described above entails the capture of host individuals. *A. astaci* occurrence in crayfish populations is tested by sampling cuticle parts from crayfish, i.e. from the soft abdominal cuticle, uropods or joints, and molecular analysis after DNA extraction of the tissue (Oidtmann et al. 2006, Vrålstad et al. 2009). Depending on size of the host population, infection prevalence and sensitivity of the detection method, the number of crayfish to be analysed for a reliable result can be high (Schrimpf et al. 2013). Crayfish plague occurrence was investigated in the years 2000-2003 and 2012 (Jean-Richard 2013) in Switzerland, but with a limited scope due to laborious conventional monitoring methods. For analysis of *B. dendrobatidis* infection status, toe-clipping, swabbing or bathing, with subsequent filtration of the bath water, of amphibian hosts have been used, followed by DNA extraction and TaqMan quantitative real-time PCR (Hyatt et al. 2007). Again, several individuals need to be captured for reliable results. A survey of *B. dendrobatidis* in *Alytes obstetricans* was conducted in the canton of Luzerne, which found that populations remained stable even in the presence of infection (Tobler et al. 2012). Monitoring of *T. bryosalmonae* is accomplished by capturing fish hosts by electrofishing, which is labour-intensive and requires careful safety precautions (e.g. Sterud et al. 2007). Moreover, histopathological examination of the kidney is required to determine the presence of PKD infection in fish. Wahli et al. (2008) conducted extensive surveys of PKD in Switzerland from 2000 to 2006, examining almost 7000 salmonids, mostly brown trout (*Salmo trutta*). *Saprolegnia parasitica* is usually determined from lesions on captured fish (Ravasi et al. 2018) or by baiting, which comprises the placement of bags containing hemp seeds into water (Ghimire et al. 2009, Rocchi et al. 2017). After high mortality outbreaks in the Doubs river, a survey for *S. parasitica* was conducted in the Doubs and connecting rivers (Paul & Belbahri 2012). For the analysis of *S. parasitica* genetic diversity within Switzerland, fish isolates were collected from different locations when signs of infection became visible, but sampling did not follow an epidemiological design (Ravasi et al. 2018).

Prior efforts for the detection of the four pathogens in water from eDNA

All four pathogens have free-swimming spore stages, which, together with the development of quantitative real-time PCR assays (Table 2), enables the application of eDNA-based methods. Such eDNA-based methods for their detection in water have been developed and applied before (see also Table 1).

Strand et al. (2011) first attempted to detect *A. astaci* in water spiked with *A. astaci* zoospores and in water of infected *P. leniusculus* (Strand et al. 2012) and later successfully detected the pathogen in lakes (Strand et al. 2014). The method was further validated against conventional detection methods and the pathogen was detected earlier than with *A. astacus* cage surveillance in an outbreak situation (Strand et al. 2019b). Since 2016, a yearly survey of *A. astaci* was conducted with eDNA-based methods in Norway (Strand et al. 2019a). A water detection method was also applied in water in aquaria experiments investigating *A. astaci* spore release dynamics in *A. astacus* and *F. limosus* (Makkonen et al. 2013, Svoboda et al. 2013). Wittwer et al. (2018b) observed seasonal patterns in *A. astaci* DNA concentrations in water that correlated with crayfish activity and further validated the method for *A. astaci* surveillance (Wittwer et al. 2019), having also compared performance of different water sampling methods (Wittwer et al. 2018a). Molecular methods to detect *B. dendrobatidis* in water were applied by Kirshtein et al. (2007), Walker et al. (2007) and Kamoroff and Goldberg (2017). Schmidt et al. (2013) and Chestnut et al. (2014) embedded *B. dendrobatidis* water detection data into an occupancy modelling framework to account for imperfect detection. In laboratory experiments, detection probabilities were examined in water of different sources (Mosher et al. 2017), while performance of water detection was compared to swabbing in the field (Mosher et al. 2018). To increase the scope and range of surveys, volunteers can be recruited for sampling, since little training is required (Julian et al. 2019). While baiting is a non-invasive technique for detection of *S. parasitica* in water, presence of the pathogen can only be confirmed after letting it grow (Ghimire et al. 2009, Rocchi et al. 2017), which makes direct detection in water much faster. After the massive fish kills due to *S. parasitica* in the Loue river, Rocchi et al. (2017) developed an eDNA detection assay for direct detection in water. Assays for detection of PKD agent *T. bryosalmonae* in water were developed and applied by Fontes et al. (2017), Carraro et al. (2018) and Hutchins et al. (2018). Data from *T. bryosalmonae* eDNA detection was incorporated into spatial network model of pathogen prevalence (Carraro et al. 2017, 2018).

The current status quo shows that direct detection of pathogens in water can be applied for disease monitoring and sometimes with better performance than conventional methods. To proceed further, methods need to be optimised to identify standards that facilitate the application of eDNA-based methods in disease management practice. For this, validating the methods in experimental facilities (Chapter 1) and by comparison to conventional methods (Chapter 2), is crucial. Knowledge gained from validation helps properly interpret survey results (Chapter 3) and identifies knowledge gaps and future avenues for improving pathogen surveillance, and, ultimately, disease management (Chapter 4).

Table 2. Real-time quantitative PCR assays used in this thesis to detect the four pathogen species in water, including primer and probe sequences, length of the target sequence and references of the published assays.

Species	Primer & Probe		Sequence (5'-3')	Target length	Ref.
Aphanomyces astaci	F	AphAstITS-39F	AAGGCTTGTGCTGGGATGTT	59	Vrålstad et al. 2009
	R	AphAstITS-97R	CTTCTTGCGAAACCTTCTGCTA		
	probe	AphAstITS-60T	TTCGGGACGACCC		
Batrachochytrium dendrobatidis	F	ITS1-3 Chytr	CCTTGATATAATACAGTGTGCCATATGTC	146	Boyle et al. 2004
	R	5.8S Chytr	AGCCAAGAGATCCGTTGTCAAA		
	probe	Chytr MGB2	CGAGTCGAACAAAAT		
Saprolegnia parasitica	F	S.p. Primer-F	AGAGCAAATCGCGGTAGTTT	127	Rocchi et al. 2017
	R	S.p. Primer-R	AGAAATGCACCAGCATACCA		
	probe	S.p. Probe-R	TGCCTTGTACTTTGACAACAGACTCGC		
Tetracapsuloides bryosalmonae	F	Tb_COI_F1q	GGTTGTTTAGTTTGGGCTCATC	103	Carraro et al. 2018
	R	Tb_COI_R1q	TCCCTGTAGGGACAGCTATTG		
	probe	Tb_probe_COI1	CAAGATCTTATTTTATGGCTGCCAC		

Thesis outline

Chapter 1: Validation of the eDNA-based detection assay in controlled experiments

The relative ease and low effort of eDNA sampling allows for extensive surveys, which can lead to sampling of locations with no previous knowledge of disease status. Therefore, eDNA results might not be confirmed by results from prior conventional surveys. Assessment of the reliability of eDNA survey results, i.e. quantifying the probability of false negative or false positive results, would increase confidence in eDNA detection results and permit their accurate interpretation. To achieve this, water samples could be taken from controlled environments with known pathogen occurrence and abundance and analysed for their performance in correctly informing about pathogen occurrence and potentially, abundance. Therefore, I conducted two experiments, first with *T. bryosalmonae*, and then with *B.*

dendrobatidis zoospores, in which water tanks filled with a known volume of water, were spiked with different estimated amounts of zoospores. To analyse the influence of inhibitors in water, half of the tanks were filled with tap water and the other half with water from a mesocosm containing leaf litter and a near-natural community of phyto- and zooplankton. I proceeded in taking water samples from the tanks using the method developed for field sampling. Pathogen detection rates and estimated DNA concentrations in water samples were compared to estimated spore concentrations in the tanks and implications of these results for eDNA surveys of those two pathogens were discussed.

Chapter 2: Comparison of an eDNA-based and a conventional detection method

Experiments as conducted in Chapter 1 provide valuable insight into processes influencing detection and quantification of pathogens with eDNA-based methods but are limited in their applicability in natural systems. Therefore, validation of the detection assay in the field by comparing it to conventional detection methods is important for accurately assessing method reliability in a realistic setting. For this, I sampled invasive crayfish populations and their ambient water for analysis of *A. astaci* occurrence in both crayfish tissue and water samples. I compared results of both methods and investigated reasons for variability within methods and ambiguity in results between methods.

Chapter 3: Survey of the four diseases in Switzerland

This chapters describes the results of the survey of the four pathogens in Switzerland. The sites were chosen to cover all major Swiss waterways and sites of interest to the cantonal authorities were included as well. The occurrence results were compared to environmental parameters, such as waterbody type, i.e. lake or river, elevation, lake or river size and river ecosystem integrity criteria. The possibilities and limitations of eDNA-based methods to survey multiple pathogens across taxonomic boundaries are discussed.

Chapter 4: Synthesis, remaining challenges and opportunities

Finally, the results of the previous chapters are revisited and discussed together. I describe possible approaches to how the method developed in this study could be further tested and improved and revisit the knowledge acquired from this thesis. Further, I identify areas of importance for future research on molecular pathogen detection in water and remaining challenges for bridging the science-practice gap.

Literature cited

Ahmed W, Huygens F, Goonetilleke A, Gardner T (2008) Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. Appl Environ Microbiol 74:5490–5496.

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. OIE Rev Sci Tech 15:603–632.

Anderson CL, Canning EU, Okamura B (1999) Molecular data implicate bryozoans as hosts for PKX (Phylum Myxozoa) and identify a clade of bryozoan parasites within the Myxozoa. Parasitology 119:555–561.

Arvanitidou M, Papa A, Constantinidis TC, Danielides V, Katsouyannopoulos V (1997) The occurrence of *Listeria* spp. and *Salmonella* spp. in surface waters. Microbiol Res 152:395–397.

Audemard C, Ragone Calvo LM, Paynter KT, Reece KS, Burreson EM (2006) Real-time PCR investigation of parasite ecology: In situ determination of oyster parasite *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. Parasitology 132:827–842.

Audemard C, Reece KS, Burreson EM (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. Appl Environ Microbiol 70:6611–6618.

Aw TG, Rose JB (2012) Detection of pathogens in water: From phylochips to qPCR to pyrosequencing. Curr Opin Biotechnol 23:422–430.

Barnes MA, Brown AD, Daum MN, de la Garza KA, Driskill J, Garrett K, Goldstein MS, Luk A, Maguire JI, Moke R, Ostermaier EM, Sanders YM, Sandhu T, Stith A, Suresh V V. (2020) Detection of the amphibian pathogens chytrid fungus (*Batrachochytrium dendrobatidis*) and ranavirus in West Texas, USA, using environmental DNA. J Wildl Dis 56:702–706.

Bass D, Stentiford GD, Littlewood DTJ, Hartikainen H (2015) Diverse Applications of Environmental DNA Methods in Parasitology. Trends Parasitol 31:499–513.

Berger L, Hyatt AD, Speare R, Longcore JE (2005) Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Dis Aquat Org 68:51–63.

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 95:9031–9036.

Bettge K, Segner H, Burki R, Schmidt-Posthaus H, Wahli T (2009a) Proliferative kidney disease (PKD) of rainbow trout: Temperature- and time-related changes of *Tetracapsuloides bryosalmonae* DNA in the kidney. Parasitology 136:615–625.

Bettge K, Wahli T, Segner H, Schmidt-Posthaus H (2009b) Proliferative kidney disease in rainbow trout: Time- and temperature-related renal pathology and parasite distribution. Dis Aquat Org 83:67–76.

Bohman P, Nordwall F, Edsman L (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. Bull Fr la Pêche la Piscic 380–381:1291–1302.

Bonadonna L, Briancesco R, La Rosa G (2019) Innovative analytical methods for monitoring microbiological and virological water quality. Microchem J 150:1–8.

Borsuk ME, Reichert P, Peter A, Schager E, Burkhardt-Holm P (2006) Assessing the decline of brown trout (*Salmo trutta*) in Swiss rivers using a Bayesian probability network. Ecol Modell 192:224–244.

Brunner JL (2020) Pooled samples and eDNA-based detection can facilitate the "clean trade" of aquatic animals. Sci Rep 10:1–11.

Burkhardt-Holm P (2002) Proliferative kidney disease: Why is it of interest for the Swiss project 'Fishnet'? J Fish Dis 25:441–442.

Canning EU, Curry A, Feist SW, Longshaw M, Okamura B (2000) A new class and order of myxozoans to accommodate parasites of bryozoans with ultrastructural observations on *Tetracapsula bryosalmonae* (PKX organism). J Eukaryot Microbiol 47:456–468.

Carraro L, Bertuzzo E, Mari L, Fontes I, Hartikainen H, Strepparava N, Schmidt-Posthaus H, Wahli T, Jokela J, Gatto M, Rinaldo A (2017) Integrated field, laboratory, and theoretical study of PKD spread in a Swiss prealpine river. Proc Natl Acad Sci USA

114:11992–11997.

Carraro L, Hartikainen H, Jokela J, Bertuzzo E, Rinaldo A (2018) Estimating species distribution and abundance in river networks using environmental DNA. Proc Natl Acad Sci USA 115:11724–11729.

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. Cell Microbiol 5:353–357.

Cerenius L, Söderhäll K, Persson M, Ajaxon R (1988) The crayfish plague fungus *Aphanomyces astaci* - Diagnosis, isolation, and pathobiology. In: *Freshwater Crayfish 7: Papers from the Seventh International Symposium on Freshwater Crayfish*. Tiefenau PG (ed) Musée Zoologique Cantonal, Lausanne, Switzerland

Chestnut T, Anderson C, Popa R, Blaustein AR, Voytek M, Olson DH, Kirshtein J (2014) Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS One 9.

Clifton-Hadley RS, Bucke D, Richards RH (1984) Proliferative kidney disease of salmonid fish: a review. J Fish Dis 7:363–377.

Cuco AP, Castro BB, Gonçalves F, Wolinska J, Abrantes N (2018) Temperature modulates the interaction between fungicide pollution and disease: Evidence from a Daphnia-microparasitic yeast model. Parasitology 145:939–947.

Cunningham AA, Daszak P, Wood JLN, Cunningham AA (2017) One Health , emerging infectious diseases and wildlife : two decades of progress ? Philos Trans R Soc B Biol Sci 372:20160167.

Daszak P, Cunningham AA, Hyatt AD (2001) Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Trop 78:103–116.

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science 287:443–449.

Daszak P, Strieby A, Cunningham AA, Longcore JE, Brown CC, Porter D (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. Herpetol J 14:201–207.

Deiner K, Bik HM, Mächler E, Seymour M, Lacoursière-Roussel A, Altermatt F, Creer S, Bista I, Lodge DM, de Vere N, Pfrender ME, Bernatchez L (2017) Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Mol Ecol 26:5872–5895.

Dunn AM, Hatcher MJ (2015) Parasites and biological invasions: Parallels, interactions, and control. Trends Parasitol 31:189–199.

Dupray E, Caprais MP, Derrien A, Fach P (1997) Salmonella DNA persistence in natural seawaters using PCR analysis. J Appl Microbiol 82:507–510.

El-Matbouli M, Hoffmann RW (2002) Influence of water quality on the outbreak of proliferative kidney disease - Field studies and exposure experiments. J Fish Dis 25:459–467.

Elmiger C, Gouskov A, Philipp U, Hertig A (2018) Flusskrebs-Managementplan Kanton Zürich. Pachtperiode 2018-2026. Zürich.

FAO (2020) The state of world fisheries and aquaculture 2020. Sustainability in action. Rome.

Feist SW, Peeler EJ, Gardiner R, Smith E, Longshaw M (2002) Proliferative kidney disease and renal myxosporidiosis in juvenile salmonids from rivers in England and Wales. J Fish Dis 25:451–458.

Fisher MC, Garner TWJ, Walker SF (2009) Global Emergence of *Batrachochytrium dendrobatidis* and Amphibian Chytridiomycosis in Space, Time, and Host. Annu Rev Microbiol 63:291–310.

Fontes I, Hartikainen H, Holland JW, Secombes CJ, Okamura B (2017) *Tetracapsuloides bryosalmonae* abundance in river water. Dis Aquat Org 124:145–157.

Freeland W (1983) Parasites and the coexistence of animal host species. Am Nat 121:223–236.

Ghimire SR, Richardson PA, Moorman GW, Lea-Cox JD, Ross DS, Hong CX (2009) An in-situ baiting bioassay for detecting phytophthora species in irrigation runoff containment basins. Plant Pathol 58:577–583.

Goldberg CS, Turner CR, Deiner K, Klymus KE, Thomsen PF, Murphy MA, Spear SF,

McKee A, Oyler-McCance SJ, Cornman RS, Laramie MB, Mahon AR, Lance RF, Pilliod DS, Strickler KM, Waits LP, Fremier AK, Takahara T, Herder JE, Taberlet P (2016) Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods Ecol Evol 7:1299–1307.

Grabner DS, El-Matbouli M (2008) Transmission of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) to *Fredericella sultana* (Bryozoa: Phylactolaemata) by various fish species. Dis Aquat Org 79:133–139.

Guy RA, Payment P, Krull UJ, Horgen PA (2003) Real-Time PCR for Quantification of Giardia and Cryptosporidium in environmental water samples and sewage. Appl Environ Microbiol 69:5178–5185.

Hall EM, Crespi EJ, Goldberg CS, Brunner JL (2016) Evaluating environmental DNA-based quantification of ranavirus infection in wood frog populations. Mol Ecol Resour 16:423–433.

Hall EM, Goldberg CS, Brunner JL, Crespi EJ (2018) Seasonal dynamics and potential drivers of ranavirus epidemics in wood frog populations. Oecologia 188:1253–1262.

Hallett SL, Bartholomew JL (2006) Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Dis Aquat Org 71:109–118.

Hallett SL, Bartholomew JL (2009) Development and application of a duplex QPCR for river water samples to monitor the myxozoan parasite *Parvicapsula minibicornis*. Dis Aquat Org 86:39–50.

Hallett SL, Ray RA, Hurst CN, Holt RA, Buckles GR, Atkinson SD, Bartholomew JL (2012) Density of the waterborne parasite *Ceratomyxa shasta* and its biological effects on salmon. Appl Environ Microbiol 78:3724–3731.

Hänfling B, Handley LL, Read DS, Hahn C, Li J, Nichols P, Blackman RC, Oliver A, Winfield IJ (2016) Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. Mol Ecol 25:3101–3119.

Hartikainen H, Johnes P, Moncrieff C, Okamura B (2009) Bryozoan populations reflect nutrient enrichment and productivity gradients in rivers. Freshw Biol 54:2320–2334.

Haugland RA, Siefring SC, Wymer LJ, Brenner KP, Dufour AP (2005) Comparison of

Enterococcus measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res 39:559–568.

Hedrick RP, Baxa D V., De Kinkelin P, Okamura B (2004) Malacosporean-like spores in urine of rainbow trout react with antibody and DNA probes to *Tetracapsuloides bryosalmonae*. Parasitol Res 92:81–88.

Hedrick RP, El-Matbouli M, Adkison MA, MacConnell E (1998) Whirling disease: Re-emergence among wild trout. Immunol Rev 166:365–376.

Hedrick RP, MacConnell E, de Kinkelin P (1993) Proliferative kidney disease of salmonid fish. Annu Rev Fish Dis 3:277–290.

Henne A, Daniel R, Schmitz RA, Gottschalk G (1999) Construction of environmental DNA libraries in *Escherichia coli* and screening for the presence of genes conferring utilization of 4- hydroxybutyrate. Appl Environ Microbiol 65:3901–3907.

Holdich DM (2003) Crayfish in Europe - an overview of taxonomy, legislation, distribution, and crayfish plague outbreaks. Proceedings of a conference held on 7th November, 2002. In: *Management & Conservation of Crayfish*. Holdich DM, Sibley PJ (eds) Bristol, p 15–34

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl Manag Aquat Ecosyst 2009:394–395.

Holm-Hansen O, Sutcliffe WH, Sharp J (1968) Measurement of deoxyribonucleic acid in the ocean and its ecological significance. Limnol Oceanogr 13:507–514.

Hutchins PR, Sepulveda AJ, Martin RM, Hopper LR (2018) A probe-based quantitative PCR assay for detecting *Tetracapsuloides bryosalmonae* in fish tissue and environmental DNA water samples. Conserv Genet Resour 10:317–319.

Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, Dalton A, Kriger K, Hero M, Hines H, Phillott R, Campbell R, Marantelli G, Gleason F, Colling A (2007) Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis*. Dis Aquat Org 73:175–192.

Hyman OJ, Collins JP (2012) Evaluation of a filtration-based method for detecting

Batrachochytrium dendrobatidis in natural bodies of water. Dis Aquat Org 97:185–195.

Jean-Richard P (2013) Krebspesterhebung in der Schweiz Kampagne 2012. BAFU, Bern:13.

Jeunen GJ, Knapp M, Spencer HG, Taylor HR, Lamare MD, Stat M, Bunce M, Gemmell NJ (2019) Species-level biodiversity assessment using marine environmental DNA metabarcoding requires protocol optimization and standardization. Ecol Evol 9:1323–1335.

Julian JT, Glenney GW, Rees C (2019) Evaluating observer bias and seasonal detection rates in amphibian pathogen eDNA collections by citizen scientists. Dis Aquat Org 134:15–24.

Kamoroff C, Goldberg CS (2017) Using environmental DNA for early detection of amphibian chytrid fungus *Batrachochytrium dendrobatidis* prior to a ranid die-off. Dis Aquat Org 127:75–79.

Karvonen A, Rintamäki P, Jokela J, Valtonen ET (2010) Increasing water temperature and disease risks in aquatic systems: Climate change increases the risk of some, but not all, diseases. Int J Parasitol 40:1483–1488.

Kent ML, Hedrick RP (1985) PKX, the causative agent of Proliferative Kidney Disease (PKD) in Pacific salmonid fishes and its affinities with the Myxozoa. J Protozool 32:254–260.

Kent ML, Higgins M, Whitaker DJ, Yokoyama H (1995) Proliferate kidney disease and *Sphaerospora oncorhynch*i in wild- caught salmonids from the Puntledge River system, Vancouver Island, British Columbia. Can J Fish Aquat Sci 52:13–17.

De Kinkelin P, Gay M, Forman S (2002) The persistence of infectivity of *Tetracapsula bryosalmonae*, infected water for rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 25:477–482.

Kirshtein JD, Anderson CW, Wood JS, Longcore JE, Voytek MA (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Dis Aquat Org 77:11–15.

Koops H, Hartmann F (1989) *Anguillicola*-infestations in Germany and in German eel

imports. J Appl Ichthyol 5:41–45.

Kouba A, Petrusek A, Kozák P (2014) Continental-wide distribution of crayfish species in Europe: Update and maps. Knowl Manag Aquat Ecosyst 413:05p1-05p31.

Lacoursière-Roussel A, Deiner K (2019) Environmental DNA is not the tool by itself. J Fish Biol:1–4.

Laramie MB, Pilliod DS, Goldberg CS (2015) Characterizing the distribution of an endangered salmonid using environmental DNA analysis. Biol Conserv 183:29–37.

Larsen MH, Mouritsen KN (2014) Temperature-parasitism synergy alters intertidal soft-bottom community structure. J Exp Mar Bio Ecol 460:109–119.

Lipp EK, Rivera ING, Gil AI, Espeland EM, Choopun N, Louis VR, Russek-Cohen E, Huq A, Colwell RR (2003) Direct detection of *Vibrio cholerae* and ctxA in Peruvian coastal water and plankton by PCR. Appl Environ Microbiol 69:3676–3680.

Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Pessier AP, Collins JP (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA 103:3165–3170.

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227.

Longshaw M, Le Deuff RM, Harris AF, Feist SW (2002) Development of proliferative kidney disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following short-term exposure to *Tetracapsula bryosalmonae* infected bryozoans. J Fish Dis 25:443–449.

Mahon AR, Horton DJ, Learman DR, Nathan LR, Jerde CL (2018) Investigating diversity of pathogenic microbes in commercial bait trade water. PeerJ 2018:1–13.

Makkonen J, Strand DA, Kokko H, Vrålstad T, Jussila J (2013) Timing and quantifying *Aphanomyces astaci* sporulation from the noble crayfish suffering from the crayfish plague. Vet Microbiol 162:750–755.

Marcogliese DJ (2008) The impact of climate change on the parasites and infectious diseases of aquatic animals.pdf. Rev Sci Tech Paris OIE (Office Int des Epizoot 27:467–484.

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M (2019) Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. Sci Rep 9:1–10.

Miaud C, Arnal V, Poulain M, Valentini A, Dejean T (2019) EDNA increases the detectability of ranavirus infection in an alpine amphibian population. Viruses 11:1–15.

Mok JS, Ryu A, Kwon JY, Kim B, Park K (2019) Distribution of *Vibrio* species isolated from bivalves and bivalve culture environments along the Gyeongnam coast in Korea: Virulence and antimicrobial resistance of *Vibrio parahaemolyticus* isolates. Food Control 106:106697.

Morris DJ, Adams A (2006) Transmission of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea), the causative organism of salmonid proliferative kidney disease, to the freshwater bryozoan *Fredericella sultana*. Parasitology 133:701–709.

Morris DJ, Adams A, Richards RH (2000) In situ hybridisation identifies the gill as a portal of entry for PKX (Phylum Myxozoa), the causative agent of proliferative kidney disease in salmonids. Parasitol Res 86:950–956.

Mosher BA, Huyvaert KP, Bailey LL (2018) Beyond the swab: ecosystem sampling to understand the persistence of an amphibian pathogen. Oecologia 188:319–330.

Mosher BA, Huyvaert KP, Chestnut T, Kerby JL, Madison JD, Bailey LL (2017) Design- and model-based recommendations for detecting and quantifying an amphibian pathogen in environmental samples. Ecol Evol 7:10952–10962.

Neish GA (1977) Observations on saprolegniasis of adult sockeye salmon, *Oncorhynchus nerka* (Walbaum). J Fish Biol 10:513–522.

Nyhlén L, Unestam T (1980) Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. J Invertebr Pathol 36:187–197.

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. J Microbiol Methods 7:57–66.

Oidtmann B, Geiger S, Steinbauer P, Culas A, Hoffmann RW (2006) Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. Dis Aquat Org 72:53–64.

Okamura B, Feist SW (2011) Emerging diseases in freshwater systems. Freshw Biol 56:627–637.

Okamura B, Hartikainen H, Schmidt-Posthaus H, Wahli T (2011) Life cycle complexity, environmental change and the emerging status of salmonid proliferative kidney disease. Freshw Biol 56:735–753.

Olson DH, Aanensen DM, Ronnenberg KL, Powell CI, Walker SF, Bielby J, Garner TWJ, Weaver G, Fisher MC (2013) Mapping the global emergence of *Batrachochytrium dendrobatidis*, the amphibian chytrid fungus. PLoS One 8.

Paul C, Belbahri L (2012) Clonalité de *Saprolegnia parasitica*, le parasite des poissons du Doubs.

Paull SH, Johnson PTJ (2011) High temperature enhances host pathology in a snail-trematode system: Possible consequences of climate change for the emergence of disease. Freshw Biol 56:767–778.

Pawlowski J, Apothéloz-Perret-Gentil L, Altermatt F (2020) Environmental (e)DNA: what's behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. Mol Ecol:mec.15643.

Peeler EJ, Oidtmann BC, Midtlyng PJ, Miossec L, Gozlan RE (2011) Non-native aquatic animals introductions have driven disease emergence in Europe. Biol Invasions 13:1291–1303.

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Can J Fish Aquat Sci 70:1123–1130.

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of Batrachochytrium dendrobatidis, a chytrid pathogen of amphibians. Mycologia 96:9–15.

Price PW, Westoby M, Rice B (1988) Parasite-mediated mompetition: some predictions and tests. 131:544–555.

Rachowicz LJ, Hero JM, Alford RA, Taylor JW, Morgan JAT, Vredenburg VT, Collins JP, Briggs CJ (2005) The novel and endemic pathogen hypotheses: Competing explanations for the origin of emerging infectious diseases of wildlife. Conserv Biol 19:1441–1448.

Ravasi D, De Respinis S, Wahli T (2018) Multilocus sequence typing reveals clonality in *Saprolegnia parasitica* outbreaks. J Fish Dis 41:1653–1665.

Richey CA, Kenelty K V., Hopkins KVS, Stevens BN, Martínez-López B, Hallett SL, Atkinson SD, Bartholomew JL, Soto E (2020) Validation of environmental DNA sampling for determination of *Ceratonova shasta* (Cnidaria: Myxozoa) distribution in Plumas National Forest, CA. Parasitol Res 119:859–870.

Richey CA, Kenelty K V, Hopkins KVS, Stevens BN, Mart B, Barnum SM, Hallett SL, Atkinson SD, Bartholomew JL, Soto E (2018) Distribution and prevalence of *Myxobolus cerebralis* in post fire areas of Plumas National Forest : Utility of environmental DNA sampling. J Aquat Anim Health 30:130–143.

Rivera ING, Lipp EK, Gil A, Choopun N, Huq A, Colwell RR (2003) Method of DNA extraction and application of multiplex polymerase chain reaction to detect toxigenic *Vibrio cholerae* O1 and O139 from aquatic ecosystems. Environ Microbiol 5:599–606.

Robert J, George E, De Jesús Andino F, Chen G (2011) Waterborne infectivity of the Ranavirus frog virus 3 in *Xenopus laevis*. Virology 417:410–417.

Robinson CV, Uren Webster TM, Cable J, James J, Consuegra S (2018) Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. Biol Conserv 222:241–252.

Rocchi S, Tisserant M, Valot B, Laboissière A, Frossard V, Reboux G (2017) Quantification of *Saprolegnia parasitica* in river water using real-time quantitative PCR: from massive fish mortality to tap drinking water. Int J Environ Health Res 27:1–10.

Roelke-Parker ME, Munson L, Packer C, Kock R, Cleaveland S, Carpenter M, O'Brien SJ, Pospischil A, Hofman-Lehmann R, Lutz H, Mwamengele GLM, Mgasa MN, Machange GA, Summers BA, Appel MJG (1996) A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). Nature 379:441–445.

Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Cloning the soil metagenome: A strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl

Environ Microbiol 66:2541–2547.

Rusch JC, Mojžišová M, Strand DA, Svobodová J, Vrålstad T, Petrusek A (2020) Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. NeoBiota 58:1–32.

Sato MO, Rafalimanantsoa A, Ramarokoto C, Rahetilahy AM, Ravoniarimbinina P, Kawai S, Minamoto T, Sato M, Kirinoki M, Rasolofo V, De Calan M, Chigusa Y (2018) Usefulness of environmental DNA for detecting *Schistosoma mansoni* occurrence sites in Madagascar. Int J Infect Dis 76:130–136.

Schets FM, Nobel PJ, Strating S, Mooijman KA, Engels GB, Brouwer A (2002) EU drinking water directive reference methods for enumeration of total coliforms and *Escherichia coli* compared with alternative methods. Lett Appl Microbiol 34:227–231.

Schmidt BR, Kéry M, Ursenbacher S, Hyman OJ, Collins JP (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: A case study of an emerging amphibian pathogen. Methods Ecol Evol 4:646–653.

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Śmietana P, Schulz R (2013) Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of European and American crayfish in central Europe. Freshw Biol 58:1116–1125.

Sengupta ME, Hellström M, Kariuki HC, Olsen A, Thomsen PF, Mejer H, Willerslev E, Mwanje MT, Madsen H, Kristensen TK, Stensgaard AS, Vennervald BJ (2019) Environmental DNA for improved detection and environmental surveillance of schistosomiasis. Proc Natl Acad Sci USA 116:8931–8940.

Shaheen MNF, Abd El-Daim SE, Ahmed NI, Elmahdy EM (2020) Environmental monitoring of Aichi virus and human bocavirus in samples from wastewater treatment plant, drain, and River Nile in Egypt. J Water Health 18:30–37.

Skerratt LF, Berger L, Clemann N, Hunter DA, Marantelli G, Newell DA, Philips A, McFadden M, Hines HB, Scheele BC, Brannelly LA, Speare R, Versteegen S, Cashins SD, West M (2016) Priorities for management of chytridiomycosis in Australia: saving frogs from extinction. Wildl Res 43:105–120.

Smith KF, Schmidt V, Rosen GE, Amaral-Zettler L (2012) Microbial diversity and potential pathogens in ornamental fish aquarium water. PLoS One 7:1–11.

Spear SF, Groves JD, Williams LA, Waits LP (2015) Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. Biol Conserv 183:38–45.

Sterud E, Forseth T, Ugedal O, Poppe TT, Jørgensen A, Bruheim T, Fjeldstad HP, Mo TA (2007) Severe mortality in wild Atlantic salmon *Salmo salar* due to proliferative kidney disease (PKD) caused by *Tetracapsuloides bryosalmonae* (Myxozoa). Dis Aquat Org 77:191–198.

Stewart JE (1991) Introductions as factors in diseases of fish and aquatic invertebrates. Can J Fish Aquat Sci 48:110–117.

Strand D, Rusch J, Tarpai A, Johnsen SI, Vrålstad T (2019a) The surveillance programme for *Aphanomyces astaci* in Norway 2018.

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: Direct monitoring approach for aquatic environments. Dis Aquat Org 95:9–17.

Strand DA, Johnsen SI, Rusch JC, Agersnap S, Larsen WB, Knudsen SW, Møller PR, Vrålstad T (2019b) Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. J Appl Ecol 56:1661–1673.

Strand DA, Jussila J, Johnsen SI, Viljamaa-Dirks S, Edsman L, Wiik-Nielsen J, Viljugrein H, Engdahl F, Vrålstad T (2014) Detection of crayfish plague spores in large freshwater systems. J Appl Ecol 51:544–553.

Strand DA, Jussila J, Viljamaa-Dirks S, Kokko H, Makkonen J, Holst-Jensen A, Viljugrein H, Vrålstad T (2012) Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. Vet Microbiol 160:99–107.

Stucki P, Zaugg B (2011) Aktionsplan Flusskrebse Schweiz. Artenförderung von Edelkrebs, Dohlenkrebs und Steinkrebs. Bern.

Stucki P, Zaugg B (2005) Decapoda. Fauna Helvetica 15. Centre Suisse de Cartographie de la Faune, Neuchâtel.

Svoboda J, Kozubíková-Balcarová E, Kouba A, Buřič M, Kozák P, Diéguez-Uribeondo J, Petrusek A (2013) Temporal dynamics of spore release of the crayfish plague pathogen from its natural host, American spiny-cheek crayfish (*Orconectes limosus*), evaluated by transmission experiments. Parasitology 140:792–801.

Taberlet P, Coissac E, Hajibabaei M, Rieseberg LH (2012) Environmental DNA. Mol Ecol 21:1789–1793.

Thalinger B, Deiner K, Harper L, Rees H, Blackman R, Sint D, Traugott M, Goldberg C, Bruce K (2020) A validation scale to determine the readiness of environmental DNA assays for routine species monitoring. PREPRINT

Thomsen PF, Willerslev E (2015) Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. Biol Conserv 183:4–18.

Thorne E, Williams ES (1988) Disease and Endangered Species: The Black-footed Ferret as a Recent Example. Conserv Biol 2:66–74.

Tobler U, Borgula A, Schmidt BR (2012) Populations of a susceptible amphibian species can grow despite the presence of a pathogenic chytrid fungus. PLoS One 7:1–8.

Toranzos GA, Alvarez AJ, Dvorsky EA (1993) Application of the polymerase chain reaction technique to the detection of pathogens in water. Water Sci Technol 27:207–210.

Tréguier A, Paillisson JM, Dejean T, Valentini A, Schlaepfer MA, Roussel JM (2014) Environmental DNA surveillance for invertebrate species: Advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. J Appl Ecol 51:871–879.

Trujillo-González A, Edmunds RC, Becker JA, Hutson KS (2019) Parasite detection in the ornamental fish trade using environmental DNA. Sci Rep 9:1–9.

Turner CR, Barnes MA, Xu CCY, Jones SE, Jerde CL, Lodge DM (2014) Particle size distribution and optimal capture of aqueous macrobial eDNA. Methods Ecol Evol 5:676–684.

Unestam T, Weiss DW (1970) The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. J Gen Microbiol 60:77–90.

Vrålstad T, Johnsen SI, Fristad RF, Edsman L, Strand D (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. Dis Aquat Org 97:75–83.

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:146–155.

Wahli T, Bernet D, Segner H, Schmidt-Posthaus H (2008) Role of altitude and water temperature as regulating factors for the geographical distribution of *Tetracapsuloides bryosalmonae* infected fishes in Switzerland. J Fish Biol 73:2184–2197.

Wahli T, Bernet D, Steiner PA, Schmidt-Posthaus H (2007) Geographic distribution of *Tetracapsuloides bryosalmonae* infected fish in Swiss rivers: An update. Aquat Sci 69:3–10.

Wahli T, Knuesel R, Bernet D, Segner H, Pugovkin D, Burkhardt-Holm P, Escher M, Schmidt-Posthaus H (2002) Proliferative kidney disease in Switzerland: Current state of knowledge. J Fish Dis 25:491–500.

Walker SF, Salas MB, Jenkins D, Garner TWJ, Cunningham AA, Hyatt AD, Bosch J, Fisher MC (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. Dis Aquat Org 77:105–112.

van West P (2006) *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 20:99–104.

Wittwer C, Nowak C, Strand DA, Vrålstad T, Thines M, Stoll S (2018a) Comparison of two water sampling approaches for eDNA-based crayfish plague detection. Limnologica 70:1–9.

Wittwer C, Stoll S, Strand D, Vrålstad T, Nowak C, Thines M (2018b) EDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia 807:87–97.

Wittwer C, Stoll S, Thines M, Nowak C (2019) EDNA-based crayfish plague detection as practical tool for biomonitoring and risk assessment of *A. astaci*-positive crayfish populations. Biol Invasions 21:1075–1088.

Chapter 1

Validation of an eDNA-based method for the detection of wildlife pathogens in water

Natalie Sieber[1,2], Hanna Hartikainen[1,2,3], Christoph Vorburger[1,2]

[1] Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

[2] ETH Zürich, Institute of Integrative Biology (IBZ), 8092 Zürich, Switzerland

[3] University of Nottingham, School of Life Sciences, Nottingham, UK

In press in Diseases of Aquatic Organisms

Abstract

Monitoring the occurrence and density of parasites and pathogens can identify high infection-risk areas and facilitates disease control and eradication measures. Environmental DNA (eDNA) techniques are increasingly used for pathogen detection due to their relative ease of application. Since many factors affect the reliability and efficacy of eDNA-based detection, rigorous validation and assessment of method limitations is a crucial first step. We evaluated an eDNA detection method using in-situ filtration of large volume water samples, developed to detect and quantify aquatic wildlife parasites by quantitative PCR (qPCR). We assessed method reliability using *Batrachochytrium dendrobatidis,* a pathogenic fungus of amphibians and the myxozoan *Tetracapsuloides bryosalmonae,* causative agent of salmonid proliferative kidney disease, in a controlled experimental setup. Different amounts of parasite spores were added to tanks containing either clean tap water or water from a semi-natural mesocosm community. Overall detection rates were higher than 80 %, but detection was not consistent among replicate samples. Within tank variation in detection emphasises the need for increased site-level replication when dealing with parasites and pathogens. Estimated parasite DNA concentrations in water samples were highly variable, and a significant increase with higher spore concentrations was observed only for *B. dendrobatidis*. Despite evidence for PCR inhibition in DNA extractions from mesocosm water samples, the type of water did not affect detection rates significantly. Direct spiking controls revealed that the filtration step reduced detection sensitivity. Our study identifies sensitive quantification and sufficient replication as major remaining challenges for eDNA-based methods for detection of parasites in water.

Keywords: aquatic parasites, environmental DNA, *Tetracapsuloides bryosalmonae, Batrachochytrium dendrobatidis,* in-situ filtration, quantitative real-time PCR

Introduction

Monitoring the occurrence and prevalence of parasites and pathogens (hereafter summarised as parasites) is crucial for identification of high infection-risk areas, as shown for example in intestinal protozoan parasites (Helmi et al. 2011) and viruses (Grewar et al. 2019). Knowledge of pathogens in the environment is also important for human health and for the mitigation and prevention of zoonoses (Cunningham et al. 2017). Furthermore, it eases planning of disease control, and even informs eradication measures, for example of *Schistosoma mansoni* in Kenya (Sengupta et al. 2019). Therefore, comprehensive and regularly updated monitoring campaigns and surveillance in order to create parasite prevalence maps (Diarra et al. 2019) are important to implement management measures effectively. However, current management of most diseases is re-active, rather than pro-active, in that disease outbreaks are often detected by chance, then triggering measures and recommendations aimed at preventing further spread of the disease, such as Ebola in West Africa (Woolhouse et al. 2015). Conventional methods of parasite monitoring are cost- and labour-intensive, further impeding regular and comprehensive monitoring campaigns for parasites in the environment.

A fungus of the Phylum Chytridiomycota, *Batrachochytrium dendrobatidis*, causative agent of chytridiomycosis, has become infamous by causing mass mortalities in amphibians worldwide (Fisher et al. 2009). It is suspected to be a major driver of the extinction of frog species (Skerratt et al. 2016) and has sparked extensive monitoring programs (Seimon et al. 2017). Conventional monitoring requires capture of sometimes rare and elusive amphibian species and taking skin swabs from the animals, before releasing them again. Fish hosts of *Tetracapsuloides bryosalmonae* (Myxozoa), causative agent of proliferative kidney disease (PKD), are less fortunate: after capture by electrofishing, pathological examination requires extraction of their kidneys (Wahli et al. 2007). *T. bryosalmonae* causes high mortality in young-of-the-year salmonid fish both in the wild and in fish farms in Europe and North America (Clifton-Hadley et al. 1984, Hedrick et al. 1993). It is, therefore, of both conservational and economic importance to closely monitor such diseases, whilst reducing the costs and conservation impacts of the surveillance itself.

As a non-invasive alternative to the described approaches, parasite detection in water samples using environmental DNA (eDNA) techniques (see Bass et al. 2015 for a review) could alleviate the aforementioned issues. Environmental DNA is defined as DNA that is not

directly retrieved from the target organism but rather from its environment and that can be in the form of dead skin cells, mucus, blood, extracellular DNA and more (Thomsen & Willerslev 2015). The same or similar eDNA techniques can be implemented for parasite detection, which, however, will mostly be detected as whole organisms, e.g. as zoospores or eggs. Research on detection in water has mostly focused on human parasites and pathogens, e.g. *Listeria* and *Salmonella* (Arvanitidou et al. 1997, Lyautey et al. 2007, Papić et al. 2019), *Legionella* (Moreno et al. 2019), and other potentially pathogenic microorganisms (Dalu et al. 2011). Furthermore, disease agents of economically important species such as *Vibrio* spp. in shellfish (Mok et al. 2019) or *Myxobolus cerebralis*, etiological agent of whirling disease in salmonids (Richey et al. 2018), are also of great interest. The advancement of molecular analytical methods has increased the potential of eDNA techniques and water sampling for parasite and pathogen detection across a broader range of systems and habitats and has been reviewed for microorganisms in drinking and recreational waters (Aw & Rose 2012, Botes et al. 2013, Bonadonna et al. 2019) and eukaryote parasites (Bass et al. 2015). Crayfish plague agent *Aphanomyces astaci* spore concentrations, derived via quantitative real-time PCR (qPCR), positively correlated with parasite prevalence in captured signal crayfish from 3 Nordic lakes (Strand et al. 2014). Rusch et al. (2018) developed and successfully applied for the first time an eDNA-based detection method of *Gyrodactylus salaris*, an ectoparasite severely damaging Atlantic salmon populations and fisheries. These studies illustrate the value that molecular, or eDNA-based, detection techniques of parasites in water add to achieve deeper understanding of parasite distribution, abundance and spread, and ultimately, implementation of management measures.

However, as eDNA-based detection methods have moved out of their infancy, limitations have become more apparent. The target species might not be detected if it is rare or exists in low densities, such as invasive species at the invasion front (Jerde et al. 2011), or even in controlled experiments (Moyer et al. 2014). This could be the case for parasites, i.e. when infection prevalence and / or intensity in the host population is low or when, as for chronic infections, parasite shedding rate is low. This can be alleviated by collecting larger sample volumes, which in turn, however, could lead to increased accumulation of inhibitory compounds (von Wintzingerode et al. 1997 and references therein). Coincidence of eDNA sampling timing with active parasite release from hosts can be crucial to maximise detection rates and, therefore, the efficacy of the method. For parasites, this requires knowledge of periods of highest transmission and proliferation, which are often seasonal, e.g. related to

water temperature (Mok et al. 2019), or can be tightly linked to host activities such as the mating season in North American crayfish (Wittwer et al. 2018). Furthermore, most parasite DNA in environmental samples is in the form of spores, cysts or eggs, which need to be physically disrupted to access the DNA, requiring rigorous extraction protocols involving multiple rounds of alternate freezing and boiling of samples (Leles et al. 2009). These aspects all affect the reliability and efficacy of eDNA-based methods for the detection of parasites in water. Rigorous validation is, therefore, crucial to assess the limitations of such methods.

In this study we validated an eDNA detection method we developed for aquatic parasites. The method consists of *in situ* filtration of large-volume samples (5 L), a DNA extraction protocol deemed efficient at removing inhibition, and qPCR analysis, which is more sensitive than endpoint PCR (Wilcox et al. 2013). The study followed four main aims. Firstly, we aimed to assess reliability of the method by investigating, in a controlled environment, the detection success of two parasite species: the amphibian chytrid fungus *B. dendrobatidis* and the PKD-causing *T. bryosalmonae*. This was achieved by adding different estimated concentrations of parasite spores to water tanks, followed by taking water samples from these tanks and measuring detection success in the water samples. The detection results were evaluated using occupancy models that estimated detection probabilities on tank, sample and qPCR replicate levels. Occupancy models can quantify the reliability of the detection on different hierarchical levels, i.e. by quantifying the probability of detection in a single water sample, given a successful detection on the level of the sampling site from which multiple water samples were taken (Dorazio & Erickson 2018). Occupancy models furthermore help in determining factors influencing detection success, such as elevation or water quality (Schmidt et al. 2013), or in our case, spore concentrations and water source. Secondly, to test the potential effect of inhibitors and to simulate more realistic conditions, half of the tanks were filled with water originating from a large mesocosm containing a semi-natural community of aquatic organisms and compared to tanks filled with tap water. Thirdly, to estimate the accuracy of the quantification with qPCR we compared estimated parasite DNA concentrations in water samples with spore concentrations in the tanks from which the samples were taken. Lastly, the effects of the filtration process and the filter on detection variability were investigated by comparisons with samples directly spiked with spores in the lab, with and without a filter. Validation of eDNA detection methods as conducted in this study are important for comparability between methods and help towards finding a consensus of which methods are most efficient and reliable for species detection in surveys.

Materials and Methods

Generation of parasite spore solutions

Zoospores of *Batrachochytrium dendrobatidis* (isolate BEW2; Farrer et al. 2013) were obtained from cultures maintained according to Longcore et al. (1999) and Longcore (2000; see Text S1 in the Supplement for a detailed protocol).To generate a solution containing *B. dendrobatidis* zoospores, culture medium was transferred to a 1.5 ml plastic tube with a pipette, taking care not to touch the bottom of the culturing flask to prevent the collection of zoosporangia. *Tetracapsuloides bryosalmonae* spores were obtained from spore sacs extracted from bryozoans *Fredericella sultana* collected in the field one day prior to the experiment (River Glane, Switzerland, September 2018). Under a microscope with x8 magnification, *T. bryosalmonae* spore sacs were carefully excised from the zooids using forceps and collected in a separate petri dish. The spore sacs were ruptured with a needle to release the spores. The solution containing spores was pipetted into a 1.5 ml plastic tube and mixed thoroughly by pipetting up and down. For both parasites, ten 6.6 µl subsamples of the solution were used to estimate spore densities microscopically using a counting chamber (KOVA Glasstic slide 10 with grids, Kova International). The mean spore concentration of both parasites was calculated from the ten subsamples and used to further dilute the solutions to 200 spores ml^{-1}, which were then stored in glass Schott flasks on ice until further usage on the same day. For dilution, clean tap water used for culturing bryozoans and fresh MGHTL culture media was utilised for *T. bryosalmonae* and *B. dendrobatidis*, respectively.

Experimental setup

The experiment with *B. dendrobatidis* was conducted on 14.11.2018 and the experiment with *T. bryosalmonae* on 18.09.2018 in a designated outdoor mesocosm facility on the roof of a laboratory building. The experiment included either 20 (*B. dendrobatidis*) or 16 (*T. bryosalmonae*) 90 L polyethylene tanks (Eurokraft Kunststoffmulde by JOPA® Kunststofftechnik S&W) filled with 20 L of either tap water or water obtained from a 1000 L mesocosm that simulated a natural pond community. The pond mesocosm was set up in May 2018. It contained tap water and local leaf litter as a source of nutrients, and it was inoculated with a townet sample of phyto- and zooplankton from nearby Lake Greifensee. In both experiments, the tanks holding 20 L of water were spiked with 100 µl, 1 ml or 10 ml of spore solution for an estimated final concentration of 1, 10 or 100 spores L^{-1} with four tanks per concentration. As a control, an additional four tanks, two for each water source, were not

spiked, to test for background parasite DNA in the water sources (n = 16 tanks in total). In the experiment with *B. dendrobatidis*, an additional treatment of 1000 spores L^{-1} in four tanks was included (n = 20 tanks in total), after the earlier experiment on *T. bryosalmonae* yielded surprisingly inconsistent detection at concentrations up to 100 spores L^{-1}. All tanks were filled with source water (mesocosm or tap) before the start of the experiment, and three 5 L subsamples were collected and filtered from each tank immediately after addition of the parasite spore spike to the tank. Prior to filtering, the spike was mixed into the tank water thoroughly by hand with clean gloves. The master spore solution was kept on ice between filtration of different tanks. In order to accomplish each of the experiments in one day, spiking and filtration were conducted for both water types in two blocks, resulting in two replicates of each water quality treatment ("mesocosm water" and "tap water") per spore concentration. Within blocks, the order of filtration was randomised. Water was filtered from each tank as it would have been in the field (Fig. 1), including a negative control at the beginning to test for cleanliness of the equipment. Sampling of one tank, including filtration of the negative control at the beginning, lasted around 45 minutes. The filters were stored on ice for a maximum of 4 h and frozen at -80°C until DNA extraction.

Sampling method

Environmental DNA samples were collected using an *in situ* filtration system with a peristaltic pump (Alexis peristaltic pump, Proactive Environmental Products LLC). Figure 1 shows a sketch of the assembled filtration system. A PVC tube (length = 2 m, ø [outer / inner] = 13 / 10 mm) is attached to a polypropylene plastic funnel (ø [outer / inner] = 100 / 95 mm) with its opening covered with a synthetic polyamide mesh with particle retention size of 100 µm (Sefar AG), to serve as an inlet for the unfiltered water. To ensure submersion, a 1 kg coated lead diving weight was attached to the funnel with a 20 cm long rope and a longer piece of rope was fastened to the weight to facilitate retrieval from the water. The PVC tube was connected to a silicone tube (75 cm long, ø [outer / inner] = 10 / 5 mm) with a plastic reducer (PP ø 8-12/4-8 mm). A filter holder (Swinnex, 47 mm, Merck Millipore) was attached to the other end of the silicone tube using two connectors (double nipple PVC ¼" x ¼" inner thread and screw-in connector GES 6 R1/4", straight 6 mm / R1/4"). A 75 cm long PVC tube (ø [outer / inner] = 10 / 6 mm) was fastened to the outlet of the filter holder to direct the filtered water to a measuring bucket (Fig. 1).

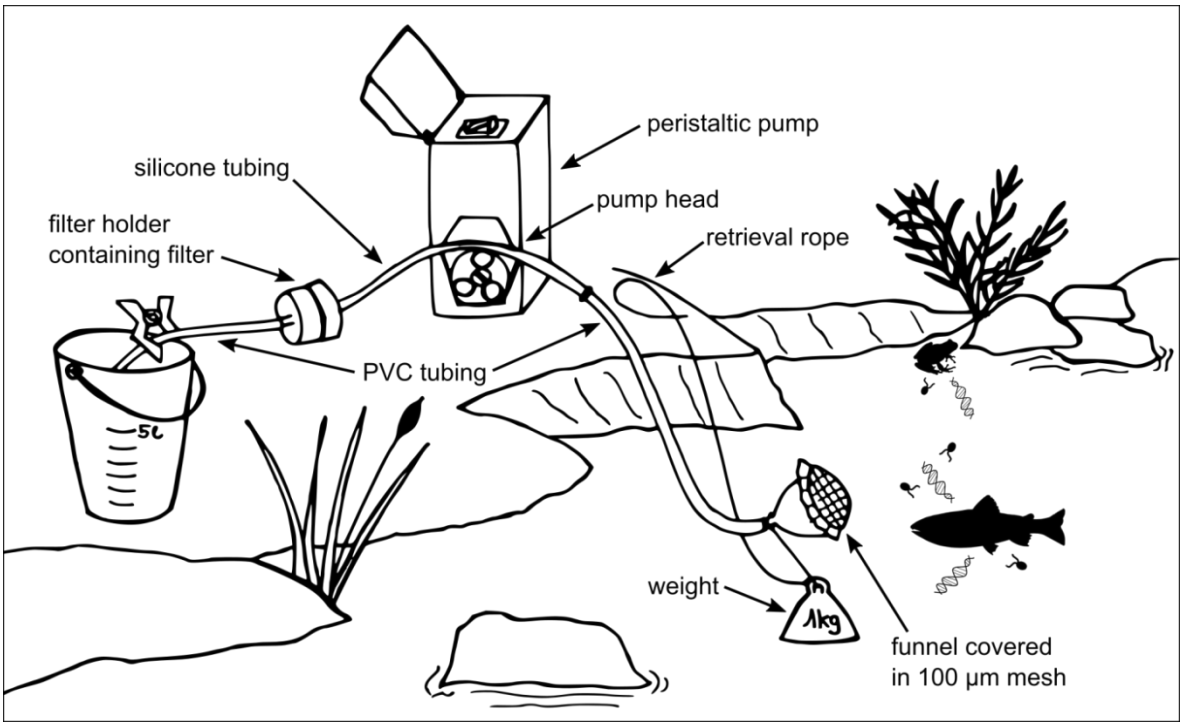


Figure 1. Sketch of the eDNA collection method developed in this study. A battery-driven peristaltic pump is used to pump water through a submerged funnel covered in a 100 µm pore-sized mesh and filtered through a glass fibre filter contained within a re-usable filter holder. Silicone tubing section compatible with the pump is extended using disposable PVC tubing to minimise contamination and requirement for cleaning.

A "site kit" was assembled and sealed under clean conditions in the lab (see Fig. S1 in the Supplement for a picture of its components). It consisted of a plastic bag containing an assembled filtering apparatus with a ready-to-use glass fibre filter with a 1 µm pore size and 47 mm diameter (Grade GF/B, Whatman, VWR) in the filter holder, a pair of plastic forceps, three glass fibre filters, four 5 ml tubes (PowerWater DNA bead tubes, Qiagen) and a pair of nitrile lab gloves. A new "site kit" was used for each experimental water tank. Parts of the equipment were cleaned and reused for the experiments since they had been used in the field before. Reused parts were the filter holder and its two O-rings, the silicone tube, all the connectors, the forceps, the funnel and the weight including the ropes. The cleaning procedure for all parts included a minimum of 10 min soak in 2.5 % diluted technical bleach (Sodium hypochlorite [14 % Cl_2] VWR, Dietikon, Switzerland), followed by a 10 min soak in de-ionised water. De-ionisation was achieved with reverse osmosis. Equipment was rinsed in MilliQ water and air-dried on a lab bench, covered with household paper. To control for the cleanliness of the filtration equipment, a negative control was run through the system

prior to sampling each tank. The negative control consisted of 5 L of MilliQ water in a flexible plastic container (Brainypack, Bottleshop, Menshen Schweiz GmbH).
Before sampling, the silicone tube was inserted into the pump head and the short PVC tube end was put into the bucket and secured in place with a clamp. Gloves included in the site kit were worn during this procedure. The negative control was then filtered, with a helper pouring the water into the funnel held by the person with the gloves. After filtration the filter holder was opened, and the filter carefully folded with the feed side inwards using forceps and put into a 5 ml tube. A new filter was inserted into the holder and the funnel was lowered into the experimental tank by the rope attached to the weight. After 5 L had been filtered the filter was exchanged as before. This procedure was repeated until three 5 L water samples were filtered, in addition to the negative control. The tubes containing the samples were stored on ice for a maximum of four hours and then frozen at -80°C until extraction.

Validation of the *T. bryosalmonae* spike consistency

In a validation experiment, the spore solution of *T. bryosalmonae* was added to pre-wetted filter papers directly, omitting the filtration step. This experiment could not be conducted with *B. dendrobatidis* due to a lack of remaining spore solution. The filters were spiked with the number of spores they would theoretically have captured in the experiment according to the spore concentrations in the tank, i.e. for 5 L from a 1 spore L^{-1} tank = 5 spores and then 50 and 500 spores, respectively, for the 10 and 100 spores L^{-1} concentrations. Filters were placed onto petri dishes, and the adequate amount of spore solution was pipetted onto the filter. Before spiking with spores, to simulate the wetness of a filter during and after the filtration process, Sigma water was added to the filter up to a total liquid (spore solution + H_2O) of 1.5 ml per filter, which was absorbed completely by the filter. Three filters per concentration were tested. To test the effect of filter presence on detection and concentrations of spiked parasite, the same number of spores was added to the DNA extractions without a filter. Due to a dwindling stock of *T. bryosalmonae* spore solution, only three samples with 5 and 50 spores were obtained without a filter.

DNA extraction

All extraction work was conducted in a dedicated lab only used for processing sensitive samples with low DNA content and for pre-PCR work. The filters were extracted following the DNEasy PowerWater Kit protocol (Qiagen AG) with the following adjustments: after

adding 1 ml of the solution PW 1 and shredding of the filter into small pieces using a pipette tip, the tubes were incubated at 65°C for 10 minutes in an oven (VWR Peqlab). After vortexing for 5 minutes at full speed on a Vortex-Genie 2 (VWR) with a 5 ml tube adapter (QIAGEN AG) samples were subjected to an additional incubation period of 10 minutes at 65°C. Samples were then centrifuged at 8000 x g with an Eppendorf 5427 R centrifuge and rotor FA-45-12-17 for 5 ml tubes (VWR). The extraction protocol used is described in detail in the Supplement Text S2. A no-template extraction control containing only a clean filter paper was included in all extraction runs. The processed samples were stored at -20°C until further analysis.

Real-time quantitative qPCR

Reaction setup and thermal cycling

The samples were analysed with real-time qPCR on a LightCycler 480 (Roche) for both parasite species. A QIAgility pipetting robot (Qiagen AG) was used for setting up the reactions. Reactions were run in triplicate. Each qPCR run included a five-fold dilution series of eight standards of a Gblocks fragment (Integrated DNA Technologies, see Text S3) with DNA concentrations ranging from 69'335 copies μl^{-1} to 0.9 copies μl^{-1} to generate a standard curve, and a negative PCR control. To detect and quantify *B. dendrobatidis* DNA in water samples we used the TaqMan assay developed by Boyle et al. (2004), which was applied to water samples before (Walker et al. 2007), using specific forward primer ITS1-3 Chytr (5'-CCTTGATATAATACAGTGTGCCATATGTC-3'), reverse primer 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3') and minor groove binding probe Chytr MGB2 (5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3'). Reactions contained 5 µl of LightCycler 480 Probes Master buffer (Roche), both primers at concentrations of 900 nM, the MGB probe at 200 nM and 2.5 µl of DNA template for a total volume of 10 µl per reaction. To detect and quantify *T. bryosalmonae* DNA in water samples we used the TaqMan assay developed by Carraro et al. (2018), which has already been used to detect *T. bryosalmonae* in water samples, with forward primer Tb_COI_F1q (5'-GGTTGTTTAGTTTGGGCTCACC-3'), reverse primer Tb_COI_R1q (5'-TCCCTGTAGGGACAGCTATTG-3') and TaqMan probe Tb_probe_COI_1 (5'-6FAM CAAGATCTTATTTTATGGCTGCCAC BHQ-1 NFQ-3'). Ten microliter reactions for *T. bryosalmonae* contained 5 µl of LightCycler 480 Probes Master buffer (Roche), forward primer at concentration of 300 nM, reverse primer at 900 nM and 250 nM for the probe and 2.5 µl of template DNA. To control for inhibition in water

samples, a synthetic template (Carraro et al. 2017) was used (5'-GTATTCCTGGTTCTGTAGGTTGAGCGTAAAACGACGGCCAGTGAATTGTAATACGACATGGTCATAGCTGTTTCCCGATACGGAAGTCCAGTCACAT -3'). This internal positive control (IPC) does not match any published sequence data. The IPC reaction setup was as follows: 5 µl of LightCycler 480 Probes Master buffer, forward primer MIMf (5'-GTATTCCTGGTTCTGTAGGTTGAGC -3') at concentration of 50 nM, reverse primer MIMr (5'- ATGTGACTGGACTTCCGTATCG -3') at 900 nM, the IPC hydrolysis probe (5'- Cy3 CGACGGCCAGTGAATTGTAATACGA BHQ-1-3') at 250 nM, 2.5 µl of water sample DNA and the IPC at a final concentration of 8.33E-19 mol L^{-1}, or 5.01833 copies $reaction^{-1}$. The IPC was run separately from the parasite assays in triplicate for each sample.

Thermal cycling for all assays started with an initial ten minutes at 95°C to activate the DNA polymerase and denature template DNA, followed by 50 cycles of 15 seconds at 95°C and one minute at 60°C. A short cooling step of ten seconds at 40°C at the end of the cycling is suggested by the manufacturer of the machine and was implemented here.

Standard curve

A double-stranded Gblocks fragment (Integrated DNA Technologies) consisting of target sequences of all assays used in this study was used to create a five-fold dilution series with 15 dilutions from concentrations of $21.67*10^7$ to 0.035 copies μl^{-1}. The Gblocks sequence information is listed in the Supplement (Text S3). For each parasite assay a qPCR run was conducted with 30 replicates each from the fifth to eleventh dilution of the initial dilution series, and 40 replicates each of Dilutions 12 to 15 (see Table S1 in the Supplement for detailed results). All replicates in each dilution were positive up to dilution 12 (100% detection). In Dilution 13, 50% of the replicates were positive for both *T. bryosalmonae* and *B. dendrobatidis* assays. Therefore, the mean cycle quantification value (Cq-value) of positive replicates of the dilution with 50 % detection (Dilution 13 = 2.22 copies $reaction^{-1}$) was defined as the limit of detection (LOD) for each parasite assay, respectively (*T. bryosalmonae* Cq = 35.646, *B. dendrobatidis* Cq = 37.706). We are aware that this limit is less stringent than the frequently used 95% detection (Bustin et al. 2009), but we deem it more appropriate when dealing with low content eDNA samples of potentially dangerous pathogens. For the sake of comparability, we applied the LOD calculator method developed by Klymus et al. (2019), which determines the LOD concentration at 95 % detection, which resulted in LOD concentrations of 10.27 copies $reaction^{-1}$ for *B. dendrobatidis* and 9.64 copies $reaction^{-1}$ for *T. bryosalmonae* for the assays used in this study. The limit of

quantification (LOQ) was defined as the concentration of the last dilution of the linear range of the standard curve, which was at 22.2 copies μl^{-1} for both parasite assays. Parameters for the standard curve were R^2 = 0.9995, slope = -3.467 and efficiency = 94.30 % for *B. dendrobatidis* and R^2 = 0.9999, slope = -3.410 and efficiency = 96.46 % for *T. bryosalmonae*. The standard curves used to define the LOD and LOQ are visualised in Fig. S2 in the Supplement for both parasites. We included Dilutions 6 ($69.33*10^3$ copies μl^{-1}) to 13 (0.89 copies μl^{-1}) on each run for absolute quantification of the DNA samples of the same run.

Data analysis

The qPCR raw data was first prepared using the LightCycler 480 Software version 1.5.1 (Roche). Cq-values were determined with the Absolute Quantification – Second Derivative Maximum method and the high confidence algorithm (LightCycler Software version 1.5.1). A water sample was considered positive if the DNA concentration of the target parasite exceeded the LOD in at least one of the three replicate qPCR reactions. Detection of the target parasite in a water tank was considered successful if at least one sample from the tank was positive for parasite DNA. Non-detections were considered as "no answer" (N.A.) and excluded from calculations of the mean. Mean Cq-values or DNA concentrations of water samples were calculated with values from positive qPCR replicates and mean values of tanks from mean values of water samples. Statistics were conducted in R version 3.6.1. (R Core Team 2019). The R package "eDNAOccupancy" (Dorazio & Erickson 2018) was used for running hierarchical occupancy models and model selection to test if spore concentrations, water source, order of sampling, block and inhibition had an effect on detection probability. All occupancy models were run with 11'000 iterations of the MCMC algorithm. Model selection was conducted using the posterior predictive loss (PPLC, Gelfand & Ghosh 1998) and Watanabe-Akaike information criterions (WAIC, Watanabe 2010). If the addition of a covariate or factor did not improve model fit, it was considered not to influence parasite DNA detection. The equation $1 - (1 - \theta)^n \geq 0.95$ was used to determine the number of water samples (n) required for successful detection probability of 95 %, with θ being the probability of detection of parasite DNA in a water sample.

Linear mixed effects models were used to test the effect of spore concentrations, water source, order of sampling and block on estimated parasite DNA concentrations, including tank and sample ID as nested random factors. Block was considered a fixed factor because it contained only two levels. After testing for model fit using AIC, order of sampling and

block effects were pooled into the residual term since they did not improve the model fit and were not significant for either *B. dendrobatidis* ($p = 0.845$, $p = 0.316$, respectively) or *T. bryosalmonae* ($p = 0.692$, $p = 0.728$, respectively).

Detection success between pipette-spiked filter and experimental samples containing the same number of spores was compared using Fisher's exact test for count data. Comparisons of parasite DNA concentrations between pipette-spiked samples with and without filter, and between pipette-spiked filter samples and experimental samples were conducted with linear models with spiked number of spores as a covariate. Significance scores of all linear models were extracted with type III analysis of variance with Satterthwaite's method.

Inhibition of water samples was quantified by calculating Cq-value differences between water samples spiked with IPC DNA and control samples containing MiliQ water spiked with IPC DNA. The larger the Cq-value differences, the more inhibited the sample, with ΔCq-values ≥ 3 signifying substantial inhibition. We tested the effect of water source on inhibition using a Wilcoxon rank sum test.

Results

Detection consistency

Batrachochytrium dendrobatidis DNA was successfully detected in 14 out of 16 spore-spiked tanks (87.5 %) and *Tetracapsuloides bryosalmonae* DNA in 10 out of 12 tanks (83 %). The two tanks where detection failed for *B. dendrobatidis* were spiked at concentrations of 1 and 100 spores L^{-1}, for *T. bryosalmonae* at 10 and 100 spores L^{-1}. Detection of parasite DNA was not consistent among samples of the same tanks. DNA of *B. dendrobatidis* was successfully detected in all three samples in only two tanks, while five tanks had two, and the rest had one DNA-positive sample (n = 7). For *T. bryosalmonae* two tanks had two positive samples out of three, while the remaining eight had only one positive sample. The mean Cq-values and concentrations are reported in full in Table S2 in the Supplement. All negative controls filtering 5 L of MilliQ water to test the cleanliness of sampling equipment (n = 36), and all samples from tanks without parasite spores (n = 24) were consistently negative.

Hierarchical occupancy models with constant parameters estimated occupancy probability in a tank to be $\Psi(.) = 0.916$ for *B. dendrobatidis* and $\Psi(.) = 0.910$ for *T. bryosalmonae*. The

estimates of DNA detection per sample were θ(.) = 0.53 and θ(.) = 0.356, respectively. Thus, to reach detection rates per tank of 95 % or above, four and seven water samples per tank need to be taken for *B. dendrobatidis* and *T. bryosalmonae*, respectively. Detection probability in a qPCR replicate, given successful parasite detection in the sample, was estimated to be p(.) = 0.665 and p(.) = 0.955, respectively. *B. dendrobatidis* model fit improved when spore concentration and water source were added as covariates at the qPCR replicate level. This result suggests that *B. dendrobatidis* DNA detection in positive samples at the level of qPCR replicates was more likely in tap water samples, p(tap water) = 0.813 vs. p(mesocosm water) = 0.553, and in tanks with higher spore concentrations, p(1 spore L^{-1}) = 0.576, p(10 spores L^{-1}) = 0.579, p(100 spores L^{-1}) = 0.608, p(1000 spores L^{-1}) = 0.843. Since none of the covariates improved *T. bryosalmonae* model fit, detection success did not depend on water source or spore concentrations per tank. All tested models are listed in Table S3 in the Supplement.

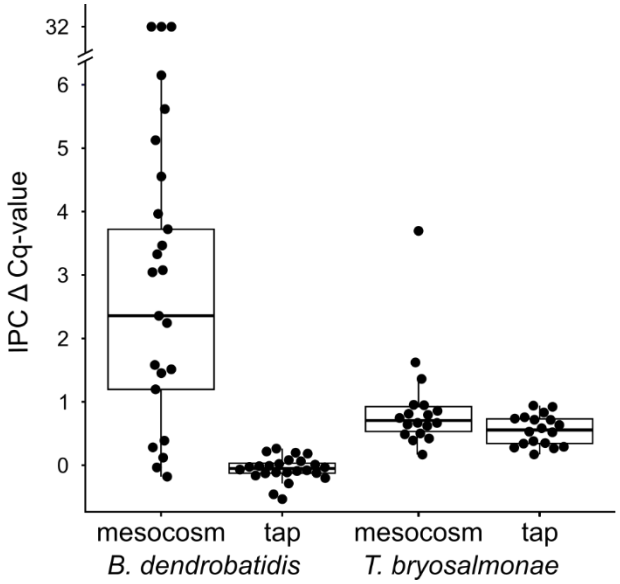


Figure 2. Internal positive control qPCR cycle differences (Δ Cq-values) between mesocosm and tap water samples of the *B. dendrobatidis* and *T. bryosalmonae* experiments. Points are jittered for better visibility. Δ Cq-values ≥ 3 indicate presence of inhibitors. The samples with Δ Cq-values > 30 had no IPC DNA amplification and therefore, Δ Cq-values were set to the Cq-value of the IPC control sample (Cq = 32.22).

Real-time qPCR inhibition

Most samples from the *B. dendrobatidis* experiment with water originating from the mesocosm (19 of 24) showed signs of inhibition according to IPC Cq-value shifts, while none of the tap water samples seemed to be affected ($p < 0.001$; Fig. 2). In contrast, only

three mesocosm water samples were affected by inhibition (Cq-value shift >3 cycles) in the *T. bryosalmonae* experiment (conducted at a different time point), and therefore, no significant effect of water source on inhibition was detected (p = 0.064).

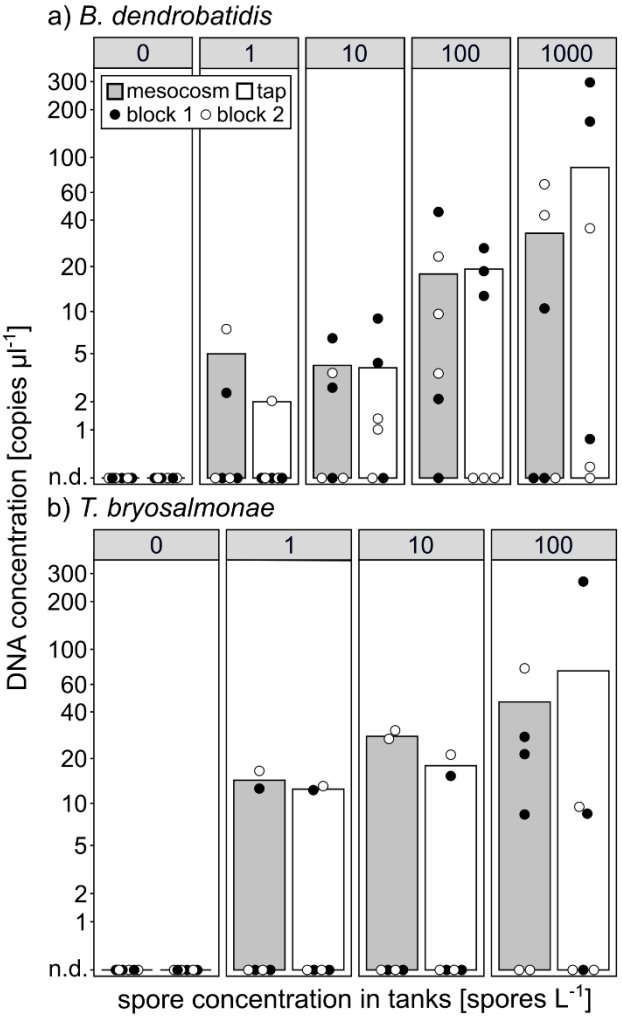


Figure 3. Mean estimated parasite DNA concentrations in copies μl^{-1} per spore concentration and water source (grey bars: mesocosm water, white bars: tap water) of a) *B. dendrobatidis* and b) *T. bryosalmonae*. To account for the large variance, concentrations are presented on a logarithmic scale. Successful detections only were used for calculating the means. The data points show mean parasite DNA concentrations of qPCR replicates per 5 L water samples (n = 6 per bar), with closed and open circles distinguishing samples from different blocks (two tanks, one in each block, per water origin and spore concentration). Points are jittered to visualise non-detections (n.d.). Parasite DNA concentrations on qPCR replicate level versus tank spore concentrations are visualised in Fig. S3 in the Supplement.

Quantification of parasite eDNA

Parasite DNA concentrations in water samples increased significantly with higher spore concentrations in tanks for *B. dendrobatidis* (F = 4.787, p = 0.041; Fig. 3a), but not *T. bryosalmonae* (F = 1.239, p = 0.29; Fig. 3b). Water source did not influence DNA concentration estimates of either parasite (F = 0.115, p = 0.738, and F = 0.09, p = 0.771, respectively). Parasite DNA concentrations on qPCR replicate level versus tank spore concentrations are visualised in Figure S3 in the Supplement.

Effect of filtration and extraction on *T. bryosalmonae* detection and quantification

T. bryosalmonae DNA was successfully detected in all replicates of samples where no filter paper was introduced. No significant differences in *T. bryosalmonae* DNA detection success between pipette-spiked filters and filters from experimental samples were observed ($p = 0.119$). No-filter samples of *T. bryosalmonae* yielded higher DNA concentration estimates than pipette-spiked filters ($F_{1,9} = 52.281$, $p < 0.001$; Fig. 4) with significant differences in concentrations depending on the number of spores per sample ($F_{1,9} = 53.785$, $p < 0.001$). The increase of *T. bryosalmonae* DNA concentration in samples with 50 spores compared to samples with five spores, was significantly different between no-filter and pipette-spiked filter samples (interaction: $F_{1,9} = 11.469$, $p = 0.008$). Pipette-spiked filter samples did not have higher *T. bryosalmonae* DNA concentrations than experimental filter samples ($F_{1,21} = 0.119$, $p = 0.733$; Fig. 4). *T. bryosalmonae* DNA concentrations of pipette-spiked filter samples significantly increased with increasing spores filter $^{-1}$ ($F_{1,8} = 27.675$, $p < 0.001$; Fig. 4). The mean Cq-values and concentrations recorded from no-filter and pipette-spiked filter samples are found in Table S4 in the Supplement.

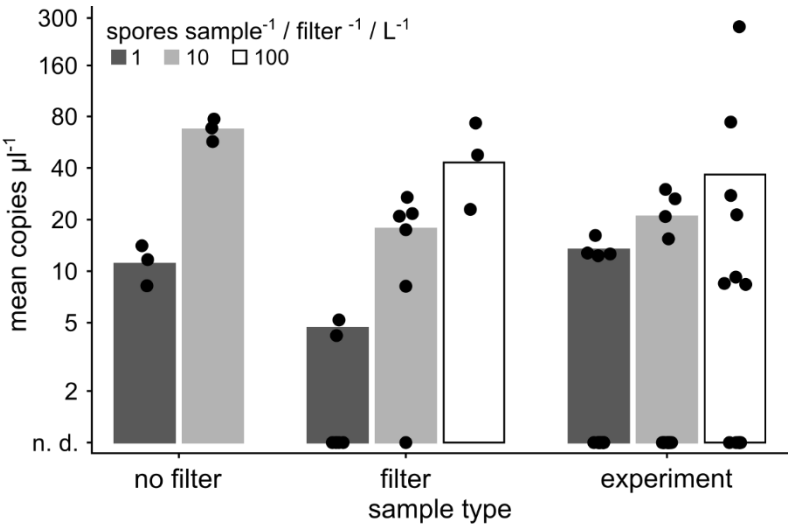


Figure 4. Mean concentrations of *T. bryosalmonae* DNA in copies µl^{-1} of no filter samples (n = 3 for each spores sample $^{-1}$ treatment), pipette-spiked samples (n = 6 each for treatments of five and 50 spores filter^{-1} and n = 3 for the 500 spores filter^{-1} treatment) and water samples of the experiment (n = 12 for each spore concentration [spores L^{-1}]). Successful detections only were used for calculating the means. Points show means of qPCR replicates for each sample and are jittered to visualise non-detections (n.d.). The data that was used to create this figure is found in Table S5 in the Supplement. *T. bryosalmonae* DNA concentrations on qPCR replicate level are visualised in Fig. S4 in the Supplement.

Discussion

Parasite detection and occupancy

The detection success of parasite spores in water using eDNA techniques was assessed in a controlled environment for a filtration method capturing 5 L water samples, developed for application in the field. We detected DNA of *Batrachochytrium dendrobatidis* in 14 out of 16, and of *Tetracapsuloides bryosalmonae* in 10 out of 12 tanks spiked with the target parasite spores. Therefore, the overall detection rate was similar for both tested parasites, i.e. 87.5 % for *B. dendrobatidis* and 83 % for *T. bryosalmonae*. However, per sample detection was far from perfect even under these controlled conditions. Sample level non-detections were observed at all spore densities of both target organisms. *B. dendrobatidis* DNA was detected in all three samples from two tanks only (2 x 100 spores L^{-1}), while most tanks yielded only one positive sample out of three (n = 7). *T. bryosalmonae* DNA was never detected in all three samples in any of the experimental tanks. Non-detections were not restricted to low concentration treatments, nor to mesocosm water where PCR inhibition was predicted to be higher (Fig. 2).

Inconsistency of detection in water samples can arise when the amount of DNA captured in a sample is low, i.e. around the LOD. We deliberately adopted a relatively permissive LOD for reporting of positive detections, in line with recommendations for low-DNA samples (Hunter et al. 2017). Since we expect most of the parasite DNA in water to originate from intact spores and not from free extracellular DNA, the encounter rate with parasite spores might not be high enough for successful detection unless they occur in sufficient densities. In our experiments, the density of spores could have been reduced through adhesion of spores to plastic or glass surfaces (tank sides, bottle lid, pipette tips, etc.), which would render them unavailable for capture via water filtration. However, *T. bryosalmonae* DNA concentrations of experimental water samples were not lower than pipette-spiked filter samples (Fig. 4), indicating that loss by adhesion in the tanks and by the filtering equipment was negligible. We note that detection rate did not increase with higher spore densities in our experiments, and even the lowest spore concentrations led to successful detections. This suggests that detection success may be compounded by other factors than low DNA concentrations, for example due to heterogeneous distribution of parasite spores in water.

Heterogeneity in the *T. bryosalmonae* experiment could have arisen by the presence of spore/sac wall clusters or by spores sticking together. Presence of spore sac fragments was

not observed during counting but cannot be completely excluded. Clumping of spores in dissected material is possible, as *T. bryosalmonae* spores contain four polar capsules, each with a polar filament used for attachment to the fish host (McGurk et al. 2005, Morris & Adams 2007), and we cannot exclude the possibility that the spores could have formed clusters post-release. Further, parasite sacs likely contained spores in different stages of maturity, whereas spores released in natural conditions from live bryozoan hosts are likely to be a more homogenous population of mature spores (the parasite sacs generally burst inside the host and only spores are ejected into the water column; Hartikainen & Okamura 2015). To further evaluate experimentally the consistency of *T. bryosalmonae* detection, inoculation with naturally released spores would be an informative addition to the quantitative results obtained here. *B. dendrobatidis* zoospores are motile and less likely to form aggregations (Berger et al. 1998). However, most spores encyst in under 24 h after their release from the zoosporangium (Berger et al. 2005) and move only short distances during that time (Piotrowski et al. 2004). The spores spiked into the tanks were of different ages. Therefore, we cannot exclude heterogeneous distribution of *B. dendrobatidis* due to formation of immobile cysts. Finally, insufficient mixing of the water could be a possible reason for heterogeneous distribution of spores in the tanks, since we only mixed the water before filtering the first sample of each tank, and the filtration of three 5 L samples took about 30 minutes. This might be enough time for spores to settle or otherwise distribute unevenly in the tank, although there was no pattern in the results suggesting this (e.g. no higher probability of detection in the first samples taken from a tank).

Many previous studies using vertebrates, e.g. fish (Klymus et al. 2015) and amphibians (Pilliod et al. 2013), or macrophytes (Gantz et al. 2018) have reported 100 % detection rates in mesocosm studies. Such promising results may arise because of the continuous and more disperse release of DNA via dead cells, mucus, etc. from these organisms. Previous studies with invertebrates and parasites in mesocosms have reported more variable detection success, in accordance with our findings (but see Sengupta et al. 2019 for 100 % detection efficiency of *Schistosoma mansoni)*. Mauvisseau et al. (2019) sampled water from mesocosms containing freshwater pearl mussels *Margaritifera margaritifera* and were able to detect their DNA in all mesocosms with only one of two assays used, with evidence for inconsistent detection in biological and technical replicates. Tapeworm *Echinococcus multilocularis* DNA was detected in all 10 L water samples spiked with 100 or 1000 *E. multilocularis* eggs, respectively, but only in two-thirds of samples spiked with 10 eggs

(Lass et al. 2019). Furthermore, while detection rate from 1 L lake water samples spiked with 10 or 1000 spores of agent of the crayfish plague, *Aphanomyces astaci*, reached almost a 100 %, single spores were detected in only 73 % of the filter samples (Strand et al. 2011).

The inconsistency of detection at water sample level is reflected in the detection probabilities estimated with occupancy models, i.e. $\theta(.) = 0.53$ and $\theta(.) = 0.356$, for *B. dendrobatidis* and *T. bryosalmonae*, respectively. However, detection consistency was higher on tank level, i.e. $\Psi(.) = 0.916$ for *B. dendrobatidis* and $\Psi(.) = 0.910$ for *T. bryosalmonae*, and qPCR replicate level, i.e. $p(.) = 0.665$ for *B. dendrobatidis* and $p(.) = 0.955$ for *T. bryosalmonae*. The former being probabilities of detecting the parasite when it is present in a tank, and the latter being probabilities of a positive detection in a replicate of a sample, given that the sample is positive. These results suggest that the highest inconsistencies in detection success occur at the sample level. According to per sample detection probabilities, a theoretical cumulative detection rate of 95 % or above can only be reached if four water samples per tank for *B. dendrobatidis* and seven samples per tank for *T. bryosalmonae*, are taken. This is despite our liberal approach of accepting a single qPCR replicate above LOD as successful detection, which we consider appropriate for low quantity eDNA samples. Taking replicate samples per site thus seems to be a requirement for reliable detection. However, increasing the number of samples leads to higher processing effort and costs, and thus requires careful consideration of costs and benefits when surveys for parasites in water are being planned.

Inhibition in water samples

We observed inhibition of the IPC amplification in water originating from the mesocosm during the *B. dendrobatidis* experiment but not during the *T. bryosalmonae* experiment (Fig. 2). In both experiments, the water came from the same mesocosm, but the experiments were separated by two months. Natural variation in the mesocosm communities (e.g. different phytoplankton densities) may thus explain the different levels of inhibition in DNA extractions between the two experiments. Larger water volumes increase the number of spores captured in a sample, but the downside of increasing volume is that it will potentially lead to higher inhibition of the samples if more inhibitory compounds accumulate. This can decrease the efficacy of eDNA-based monitoring methods (Harper et al. 2019). In our study, water source, and therefore inhibition, did not influence detection success on tank level of either parasite in our tank experiment (Fig. 3). However, *B. dendrobatidis* mesocosm water samples did have lower detection consistency on the qPCR replicate level than tap water

samples (p(mesocosm) = 0.553, p(tap) = 0.813), indicating a potential inhibition effect. This was not the case for *T. bryosalmonae* samples, which might explain the higher overall qPCR replicate detection probability of *T. bryosalmonae* (p(.) = 0.955) compared to *B. dendrobatidis* (p(.) = 0.665). No direct effect of the magnitude of inhibition (measured shift in IPC Cq-value between a clean water and experimental DNA extract) on detection success was indicated by the statistical analysis. It could be that the low amount of IPC template added to qPCR reactions (5 copies reaction^{-1}) renders the IPC inhibition control more sensitive to inhibition than the parasite detection assays.

Accuracy of quantification

Parasite DNA concentration estimates in water samples increased with spore concentrations in tanks (Fig. 3). This relationship was statistically significant for *B. dendrobatidis* (Fig. 3a), and a similar trend is visible for *T. bryosalmonae* (Fig. 3b), even though it is not statistically significant. The difference likely resulted from the fact that the *B. dendrobatidis* experiment included tank concentrations of 1000 spores L^{-1}, while the maximum concentration in the *T. bryosalmonae* experiment was 100 spores L^{-1}. Even though one correlation was statistically significant, the large variance in the data, especially in samples from tanks with high parasite concentrations, urges caution in interpreting the results quantitatively. An accurate quantification of parasite DNA concentrations is not fulfilled by the method used in this study, according to the data. A qualitative comparison of parasite spore abundance might be possible between sites, if enough water samples are taken. Correlations between species densities and eDNA quantities has been previously found for amphibians (Thomsen et al. 2012, Pilliod et al. 2013), crayfish (Harper et al. 2018) and freshwater snails (Mauvisseau et al. 2019) in mesocosms. Infection prevalence has been shown to positively correlate or temporally coincide with DNA concentrations in water of amphibian (Huver et al. 2015), crayfish (Strand et al. 2014), fish (Hallett et al. 2012) and human parasites (Wade et al. 2010). However, a precise quantitative relationship of eDNA concentrations in water and parasite densities is yet to be described.

Influence of filtration methodology on detection success

All qPCR replicates of *T. bryosalmonae* spore samples extracted without a glass fibre filter detected parasite DNA and yielded higher DNA concentration estimates compared to the same number of spores extracted from filters (Fig. 4). *T. bryosalmonae* detection success of

pipette-spiked filter samples did not differ from experimental samples employing the same filter type. Thus, glass fibre filters, rather than the filtration process, seem to have decreased DNA yield. Filtering has been previously shown to result in weaker qPCR signals during parasite detection (Hallett & Bartholomew 2006). While glass fibre filters have been shown to work well for eDNA studies (Eichmiller et al. 2016, Spens et al. 2017), in general the optimal filter paper choice may vary according to the study objectives (Djurhuus et al. 2017, Deiner et al. 2018). Glass fibre filters are absorbent and retain more of the lysate containing DNA than some other filter types, potentially decreasing yield of target parasite DNA. Glass fibre filters (GF/B) employed in this study allow a large volume of water to be filtered, which is potentially important for detection of relatively rare parasite spores in the environment. We did not test the effect of sample volume on detection efficiency, but it seems unlikely that detection of one spore L^{-1} could be consistently achieved with smaller sample sizes. Thus, the choice of filter type and volume of water sampled presents a trade-off that is perhaps of more importance in parasite detection than for eDNA studies in general.

Implications for parasite detection in the field

The inconsistent detection we observed when applying the method in a very simplified environment is rather sobering and contains an important message for real surveys: patchiness in detection is likely to be inherent to any experimental setting where parasites are assayed in subsamples taken from rivers or lakes. Knowledge of *B. dendrobatidis* and *T. bryosalmonae* densities in natural systems, or parasite release dynamics from host individuals, is sparse and difficult to estimate outside the laboratory settings (Maguire et al. 2016, Fontes et al. 2017). The range of spore concentrations tested in this experiment likely encompasses at least some of the concentrations encountered in the field. However, the influence of spatial and temporal fluctuations on spore densities is difficult to capture. Natural aquatic systems are complex in their hydrology and structure, and thus, heterogeneous distribution and density of parasite spores and DNA, even on small scales, are expected (Shogren et al. 2017). This will particularly be the case in systems with low host abundance or parasite prevalence in the host, or where the exact sampling location and timing of sampling determines occurrence of parasite spores in a water sample. In this context, the overall detection rates obtained in this experiment were still high (87.5 and 83% for *B. dendrobatidis* and *T. bryosalmonae*, respectively), thanks to replicated sampling and technical replication within samples. Notably, low spore densities down to one spore L^{-1}

could still be detected, while no false positives were found in our study. For assessment of disease risk, the distinction between viable and non-viable parasites is important and poses additional challenges for interpretation and application of monitoring results in management.

Conclusions

This study presents a validation of an eDNA-based method to detect parasite DNA in water. An *in situ* filtration method was applied to water spiked with known amounts of spores in a controlled environment. This validation identified limits and remaining challenges of such monitoring methods, which can therefore be addressed and improved. Specifically, it showed that despite the high sensitivity of qPCR assays, detection can be very inconsistent even at high spore concentrations, presumably due to non-homogeneous spore distributions and variation introduced at the different processing steps (i.e. filtration and DNA extraction from filters). An awareness of the limitations of the method helps us to interpret the results from field surveys more adequately and to quantify uncertainties. In the present case, it highlights the need for sufficient replication of samples from the same collection site to maximise detection success. While we did observe a positive correlation between estimated DNA concentrations and the manipulated spore concentrations, the large variance of the data compromises the reliability of quantitative comparisons. In general, we advocate further studies that evaluate the feasibility of quantifying parasite loads in water at a level of accuracy that is relevant for risk assessments and early warning systems of disease outbreaks. Our 5 L filtration method was equally efficient at detecting 1 spore L^{-1} or 100 spores L^{-1}, suggesting high sensitivity, albeit the patchy occurrence of false negatives. Studies such as ours can help practitioners decide which detection method to use for monitoring campaigns. Therefore, the development of standardised validation practices for eDNA-based methods used in species detection is an important step still to be undertaken by researchers to facilitate their widespread implementation. As a step further, field-based evaluations of detection success are recommended to obtain the most relevant guidance for monitoring campaign designs.

Acknowledgements We thank Trenton Garner, Lola Brookes and Celia Serrano of the Zoological Society of London, and Matthew Fisher and Claudia Wierzbicki of Imperial College London for providing *Batrachochytrium dendrobatidis* DNA extracts for testing the

qPCR assay, *B. dendrobatidis* isolates and instructions for culturing. We further thank Raffael Stegmayer, Bettina Dubach, Nora Weissert, Tamara Schlegel and Thomas Müller for their assistance in conducting the experiments. The research was supported by a grant from Eawag and by funds from ETH Zürich and the University of Applied Sciences and Arts Northwestern Switzerland (FHNW).

Literature cited

Arvanitidou M, Papa A, Constantinidis TC, Danielides V, Katsouyannopoulos V (1997) The occurrence of *Listeria* spp. and *Salmonella* spp. in surface waters. Microbiol Res 152:395–397

Aw TG, Rose JB (2012) Detection of pathogens in water: from phylochips to qPCR to pyrosequencing. Curr Opin Biotechnol 23:422–430

Bass D, Stentiford GD, Littlewood DTJ, Hartikainen H (2015) Diverse applications of environmental DNA methods in parasitology. Trends Parasitol 31:499–513

Berger L, Hyatt AD, Speare R, Longcore JE (2005) Life cycle stages of the amphibian chytrid *Batrachochytrium dendrobatidis*. Dis Aquat Org 68:51–63

Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, Slocombe R, Ragan MA, Hyatt AD, McDonald KR, Hines HB, Lips KR, Marantelli G, Parkes H (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proc Natl Acad Sci USA 95:9031–9036

Bonadonna L, Briancesco R, La Rosa G (2019) Innovative analytical methods for monitoring microbiological and virological water quality. Microchem J 150:104160

Botes M, De Kwaadsteniet M, Cloete TE (2013) Application of quantitative PCR for the detection of microorganisms in water. Anal Bioanal Chem 405:91–108

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T,

Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Carraro L, Bertuzzo E, Mari L, Fontes I, Hartikainen H, Strepparava N, Schmidt-Posthaus H, Wahli T, Jokela J, Gatto M, Rinaldo A (2017) Integrated field, laboratory, and theoretical study of PKD spread in a Swiss prealpine river. Proc Natl Acad Sci USA 114:11992–11997

Carraro L, Hartikainen H, Jokela J, Bertuzzo E, Rinaldo A (2018) Estimating species distribution and abundance in river networks using environmental DNA. Proc Natl Acad Sci USA 115:11724–11729

Clifton-Hadley RS, Bucke D, Richards RH (1984) Proliferative kidney disease of salmonid fish: a review. J Fish Dis 7:363–377

Cunningham AA, Daszak P, Wood JLN, Cunningham AA (2017) One Health , emerging infectious diseases and wildlife : two decades of progress ? Philos Trans R Soc B Biol Sci 372:20160167

Dalu T, Barson M, Nhiwatiwa T (2011) Impact of intestinal microorganisms and protozoan parasites on drinking water quality in Harare, Zimbabwe. J Water Sanit Hyg Dev 1:153–163

Deiner K, Lopez J, Bourne S, Holman L, Seymour M, Grey EK, Lacoursière A, Li Y, Renshaw MA, Pfrender ME, Rius M, Bernatchez L, Lodge DM (2018) Optimising the detection of marine taxonomic richness using environmental DNA metabarcoding: the effects of filter material, pore size and extraction method. Metabarcoding and Metagenomics 2:1–15

Diarra B, Diarra M, Diall O, Bass B, Sanogo Y, Coulibaly E, Sylla M, Zhao W, Paone M, Cecchi G (2019) A national atlas of tsetse and African animal trypanosomosis in Mali. Parasites and Vectors 12:466

Djurhuus A, Port J, Closek CJ, Yamahara KM, Romero-Maraccini O, Walz KR, Goldsmith DB, Michisaki R, Breitbart M, Boehm AB, Chavez FP (2017) Evaluation of filtration and DNA extraction methods for environmental DNA biodiversity assessments across multiple trophic levels. Front Mar Sci 4:314

Dorazio RM, Erickson RA (2018) Ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380

Eichmiller JJ, Miller LM, Sorensen PW (2016) Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. Mol Ecol Resour 16:56–68

Farrer RA, Henk DA, Garner TWJ, Balloux F, Woodhams DC, Fisher MC (2013) Chromosomal copy number variation, selection and uneven rates of recombination reveal cryptic genome diversity linked to pathogenicity. PLoS Genet 9:e1003703

Fisher MC, Garner TWJ, Walker SF (2009) Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annu Rev Microbiol 63:291–310

Fontes I, Hartikainen H, Holland JW, Secombes CJ, Okamura B (2017) *Tetracapsuloides bryosalmonae* abundance in river water. Dis Aquat Org 124:145–157

Gantz CA, Renshaw MA, Erickson D, Lodge DM, Egan SP (2018) Environmental DNA detection of aquatic invasive plants in lab mesocosm and natural field conditions. Biol Invasions 20:2535–2552

Gelfand AE, Ghosh SK (1998) Model choice: a minimum posterior predictive loss approach. Biometrika 85:1–11

Grewar JD, Sergeant ES, Weyer CT, van Helden LS, Parker BJ, Anthony T, Thompson PN (2019) Establishing post-outbreak freedom from African horse sickness virus in South Africa's surveillance zone. Transbound Emerg Dis 66:2288–2296

Hallett SL, Bartholomew JL (2006) Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Dis Aquat Org 71:109–118

Hallett SL, Ray RA, Hurst CN, Holt RA, Buckles GR, Atkinson SD, Bartholomew JL (2012) Density of the waterborne parasite *Ceratomyxa shasta* and its biological effects on salmon. Appl Environ Microbiol 78:3724–3731

Harper KJ, Anucha NP, Turnbull JF, Bean CW, Leaver MJ (2018) Searching for a signal: Environmental DNA (eDNA) for the detection of invasive signal crayfish,

Pacifastacus leniusculus (Dana, 1852). Manag Biol Invasions 9:137–148

Harper LR, Buxton AS, Rees HC, Bruce K, Brys R, Halfmaerten D, Read DS, Watson H V, Sayer CD, Jones EP, Priestley V, Ma E, Medupin C, Burgess K, Garce S, Benson G, Boonham N, Griffiths RA, Lawson L (2019) Prospects and challenges of environmental DNA (eDNA) monitoring in freshwater ponds. Hydrobiologia 826:25–41

Hartikainen H, Okamura B (2015) Ecology and Evolution of Malacosporean-Bryozoan Interaction. In: Okamura B, Gruhl A, Bartholomew JL (eds) Myxozoan Evolution, Ecology and Development. Springer International Publishing, Basel p 201–216

Hedrick RP, MacConnell E, de Kinkelin P (1993) Proliferative kidney disease of salmonid fish. Annu Rev Fish Dis 3:277–290

Helmi K, Skraber S, Burnet JB, Leblanc L, Hoffmann L, Cauchie HM (2011) Two-year monitoring of *Cryptosporidium parvum* and *Giardia lamblia* occurrence in a recreational and drinking water reservoir using standard microscopic and molecular biology techniques. Environ Monit Assess 179:163–175

Hunter ME, Dorazio RM, Butterfield JSS, Meigs-Friend G, Nico LG, Ferrante JA (2017) Detection limits of quantitative and digital PCR assays and their influence in presence – absence surveys of environmental DNA. Mol Ecol Resour 17:221–229

Huver JR, Koprivnikar J, Johnson PTJ, Whyard S (2015) Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol Appl 25:991–1002

Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) 'Sight-unseen' detection of rare aquatic species using environmental DNA. Conserv Lett 4:150–157

Klymus KE, Merkes CM, Allison MJ, Jackson CA, Goldberg CS, Helbing CC, Hunter ME, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter CA (2019) Reporting the limits of detection and quantification for environmental DNA assays. Environ DNA 00:1–12

Klymus KE, Richter CA, Chapman DC, Paukert C (2015) Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp

Hypophthalmichthys molitrix. Biol Conserv 183:77–84

Lass A, Szostakowska B, Kontogeorgos I, Korzeniewski K, Karamon J, Sulima M, Karanis P (2019) First detection of *Echinococcus multilocularis* in environmental water sources in endemic areas using capsule filtration and molecular detection methods. Water Res 160:466–474

Leles D, Araújo A, Vicente ACP, Iñiguez AM (2009) Molecular diagnosis of ascariasis from human feces and description of a new *Ascaris* sp. genotype in Brazil. Vet Parasitol 163:167–170

Longcore JE (2000) Culture technics for amphibian chytrids: recognizing, isolating and culturing *Batrachochytrium dendrobatidis* from amphibians. In: *Proceedings of Getting the Jump! On Amphibian Diseases Conference / Workshop*. James Cook University, Cairns, Australia, p 52–54

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227

Lyautey E, Lapen DR, Wilkes G, McCleary K, Pagotto F, Tyler K, Hartmann A, Piveteau P, Rieu A, Robertson WJ, Medeiros DT, Edge TA, Gannon V, Topp E (2007) Distribution and characteristics of *Listeria monocytogenes* isolates from surface waters of the South Nation River watershed, Ontario, Canada. Appl Environ Microbiol 73:5401–5410

Maguire C, DiRenzo G V., Tunstall TS, Muletz CR, Zamudio KR, Lips KR (2016) Dead or alive? Viability of chytrid zoospores shed from live amphibian hosts. Dis Aquat Org 119:179–187

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M (2019) Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. Sci Rep 9:580

McGurk C, Morris DJ, Bron JE, Adams A (2005) The morphology of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) spores released from *Fredericella sultana* (Bryozoa: Phylactolaemata). J Fish Dis 28:307–312

Mok JS, Ryu A, Kwon JY, Kim B, Park K (2019) Distribution of *Vibrio* species isolated

from bivalves and bivalve culture environments along the Gyeongnam coast in Korea: virulence and antimicrobial resistance of *Vibrio parahaemolyticus* isolates. Food Control 106:106697

Moreno Y, Moreno-Mesonero L, García-Hernández J (2019) DVC-FISH to identify potentially pathogenic *Legionella* inside free-living amoebae from water sources. Environ Res 176:108521

Morris DJ, Adams A (2007) Sacculogenesis and sporogony of *Tetracapsuloides bryosalmonae* (Myxozoa: Malacosporea) within the bryozoan host *Fredericella sultana* (Bryozoa: Phylactolaemata). Parasitol Res 100:983–992

Moyer GR, Díaz-Ferguson E, Hill JE, Shea C (2014) Assessing environmental DNA detection in controlled lentic systems. PLoS One 9:e103767

Papić B, Golob M, Kušar D, Pate M, Zdovc I (2019) Source tracking on a dairy farm reveals a high occurrence of subclinical mastitis due to hypervirulent *Listeria monocytogenes* clonal complexes. J Appl Microbiol 127:1349–1361

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Can J Fish Aquat Sci 70:1123–1130

Piotrowski JS, Annis SL, Longcore JE (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9–15

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.r-project.org/

Richey CA, Kenelty K V, Hopkins KVS, Stevens BN, Mart B, Barnum SM, Hallett SL, Atkinson SD, Bartholomew JL, Soto E (2018) Distribution and prevalence of *Myxobolus cerebralis* in post fire areas of Plumas National Forest : utility of environmental DNA sampling. J Aquat Anim Health 30:130–143

Rusch JC, Hansen H, Strand DA, Markussen T, Hytterød S, Vrålstad T (2018) Catching the fish with the worm: A case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Parasites and Vectors 11:333

Schmidt BR, Kéry M, Ursenbacher S, Hyman OJ, Collins JP (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. Methods Ecol Evol 4:646–653

Seimon TA, Seimon A, Yager K, Reider K, Delgado A, Sowell P, Tupayachi A, Konecky B, Mcaloose D, Halloy S (2017) Term monitoring of tropical alpine habitat change, Andean anurans, and chytrid fungus in the Cordillera Vilcanota, Peru: results from a decade of study. Ecol Evol 7:1527–1540

Sengupta ME, Hellström M, Kariuki HC, Olsen A, Thomsen PF, Mejer H, Willerslev E, Mwanje MT, Madsen H, Kristensen TK, Stensgaard AS, Vennervald BJ (2019) Environmental DNA for improved detection and environmental surveillance of schistosomiasis. Proc Natl Acad Sci USA 116:8931–8940

Shogren AJ, Tank JL, Andruszkiewicz E, Olds B, Mahon AR, Jerde CL, Bolster D (2017) Controls on eDNA movement in streams: transport, retention, and resuspension. Sci Rep 7:5065

Skerratt LF, Berger L, Clemann N, Hunter DA, Marantelli G, Newell DA, Philips A, McFadden M, Hines HB, Scheele BC, Brannelly LA, Speare R, Versteegen S, Cashins SD, West M (2016) Priorities for management of chytridiomycosis in Australia: saving frogs from extinction. Wildl Res 43:105–120

Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SST, Sigsgaard EE, Hellström M (2017) Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods Ecol Evol 8:635–645

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Dis Aquat Org 95:9–17

Strand DA, Jussila J, Johnsen SI, Viljamaa-Dirks S, Edsman L, Wiik-Nielsen J, Viljugrein H, Engdahl F, Vrålstad T (2014) Detection of crayfish plague spores in large freshwater systems. J Appl Ecol 51:544–553

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012) Detection of a diverse marine fish fauna using environmental DNA from Seawater

Samples. PLoS One 7:e41732

Thomsen PF, Willerslev E (2015) Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. Biol Conserv 183:4–18

von Wintzingerode F, Göbel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213–229

Wade TJ, Sams E, Brenner KP, Haugland R, Chern E, Beach M, Wymer L, Rankin CC, Love D, Li Q, Noble R, Dufour AP (2010) Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. Environ Heal A Glob Access Sci Source 9:1–14

Wahli T, Bernet D, Steiner PA, Schmidt-Posthaus H (2007) Geographic distribution of *Tetracapsuloides bryosalmonae* infected fish in Swiss rivers: An update. Aquat Sci 69:3–10

Walker SF, Salas MB, Jenkins D, Garner TWJ, Cunningham AA, Hyatt AD, Bosch J, Fisher MC (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. Dis Aquat Org 77:105–112

Watanabe S (2010) Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. J Mach Learn Res 11:3571–3594

Wilcox TM, McKelvey KS, Young MK, Jane SF, Lowe WH, Whiteley AR, Schwartz MK (2013) Robust detection of rare species using environmental DNA: the importance of primer specificity. PLoS One 8:e59520

Wittwer C, Stoll S, Strand D, Vrålstad T, Nowak C, Thines M (2018) EDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia 807:87–97

Woolhouse MEJ, Rambaut A, Kellam P (2015) Lessons from Ebola : improving infectious disease surveillance to inform outbreak management. Sci Transl Med 7: 307rv5

Supplementary Material Chapter 1

Text S1. *Batrachochytrium dendrobatidis* culturing and maintenance protocol

Autoclaved culture media mGThL (0.8% tryptone, 0.2% gelatin hydrolysate, 0.4% lactose), stored at 4°C, was warmed to 18°C before use. For culture passaging, 10 ml mGThL were transferred to a new 25 ml Nunc flask (Nunc EasYFlasks, Nunclon ∆ Surface, Merck AG, Zug, Switzerland) using a sterile 25 ml serological pipette (Costar® Stripette®, Corning Inc., Corning NY, USA). A sterile 1 ml plastic Pasteur pipette (Pastette®, Alpha Laboratories, Eastleigh, UK) was used to scrape approximately 1cm^2 from the bottom of the old culture flask and to transfer approximately 1 ml of zoospore suspension into the new flask. Newly passaged cultures were kept at 18°C for three to seven days before being transferred to 4°C for long-term storage. Cultures were re-passaged every month for a year after being obtained, and every three months after that. Survival was checked regularly using a microscope with 10x10 and 10x40 magnification, before and after re-passaging. All the procedures were conducted in a biosafety cabinet.

Text S2. Adjusted DNeasy PowerWater extraction protocol for eDNA samples

1. Add 1ml of solution PW1 to the PW DNA bead tube.

2. Use pipette tip to break up the filter

3. Heat tubes at 65°C for 10 min.

4. Secure the tubes horizontally to a vortex adapter.

5. Vortex at max. speed for 5 min.

6. Heat tubes at 65°C for 10 min.

7. Centrifuge the tubes 8000 x g for 1 min at RT.

8. Transfer the supernatant to a clean 2ml collection tube. Draw up the supernatant by placing the pipette tip into the beads (required).

9. Centrifuge at 13'000 x g for 1 min at RT.

10. Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube.

11. Add 200 ul of Solution IRS and vortex briefly to mix. Incubate at 4°C for 5 min.

12. Centrifuge at 13'000 x g for 1 min at RT.

13. Avoiding the pellet, transfer the supernatant to a clean 2 ml collection tube.

14. Add 650 ul of Solution PW3 and vortex briefly to mix.

15. Load 650 ul of supernatant onto a MB Spin Column Centrifuge at 13'000 x g for 1 min.

16. Discard the flow-through. Repeat until all the supernatant has been processed.

17. Place the MB Spin Column into a clean 2 ml collection tube.

18. Add 650 ul of Solution PW4 (shake before use). Centrifuge at 13'000 x g for 1 min.

19. Discard the flow-through and add 650 ul of ethanol and centrifuge at 13'000 x g for 1 min.

20. Discard the flow-through and centrifuge again at 13'000 x g for 2 min.

21. Place the MB Spin Column into a clean 2 ml collection tube.

22. Add 100 ul of Solution EB to the center of the white filter membrane.

23. Centrifuge at 13'000 x g for 1 min.

24. Discard the MB Spin Column. The DNA is now ready for downstream applications.

Text S3. Sequence of double-stranded Gblocks fragment used to create the standard curve for quantitative PCR. Forward and reverse primers are marked in **bold**, probes in **bold** and *italic* for *B. dendrobatidis* and *T. bryosalmonae* (highlighted in grey).

5'-
AAGGCTTGTGCTGGGATGTTCTTCGGGACGACCCGGCTAGCAGAAGGTTTCGCAAGAAGTTTT
T**CCTTGATATAATACAGTGTGCCATATGTC**A***CGAGTCGAACAAAAT***TTATTTATTTTTTCGACA
AATTAATTGGAAATTGAATAATTTAATTGAAAAAAATTGAAAATAAATATTAAAAACAACT**TTTGA**
CAACGGATCTCTTGGCTTTTTTAGAGCAAATCGCGGTAGTTTTGCTTGTACTTCGGTACGAGT
GGACACATATTGCTTTTTGTGATTTCTGCGAGTCTGTTGTCAAAGTACAAGGCACGTAAGGAGA
GTTGGTATGCTGGTGCATTTCTTTTTT**GGTTGTTTAGTTTGGGCTCACC**ATATGTATGTTGTTG
GTTTAGACACTGATAC***AAGATCTTATTTTATGGCTGCCAC***TATGA**CAATAGCTGTCCCTACAG**
GGA -3'

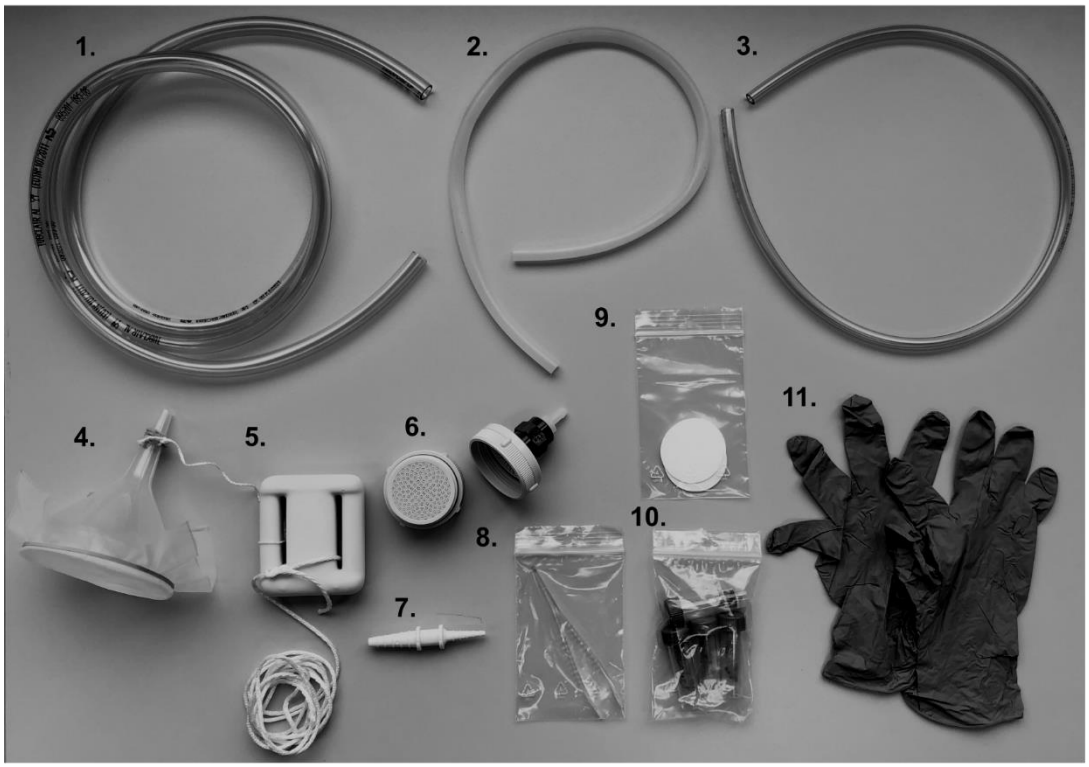


Figure S1. Components of sampling site kit: 1. PVC tube (ø (outer / inner) = 13 / 10 mm), 2. silicone tube (ø (outer / inner) = 10 / 5 mm), 3. PVC tube (ø (outer / inner) = 10 / 6 mm), 4. plastic funnel (ø (outer / inner) = 100 / 95 mm) with synthetic mesh (particle retention: 100 µm), 5. diving weight (1 kg) attached to retrieval rope, 6. filter holder (Swinnex, ø = 47 mm), 7. plastic reducer (PP ø 8-12/4-8 mm), 8. plastic forceps, 9. glass fibre filters (Grade GF/B, ø = 47 mm), 10. PowerWater DNA bead tubes, 11. nitrile gloves.

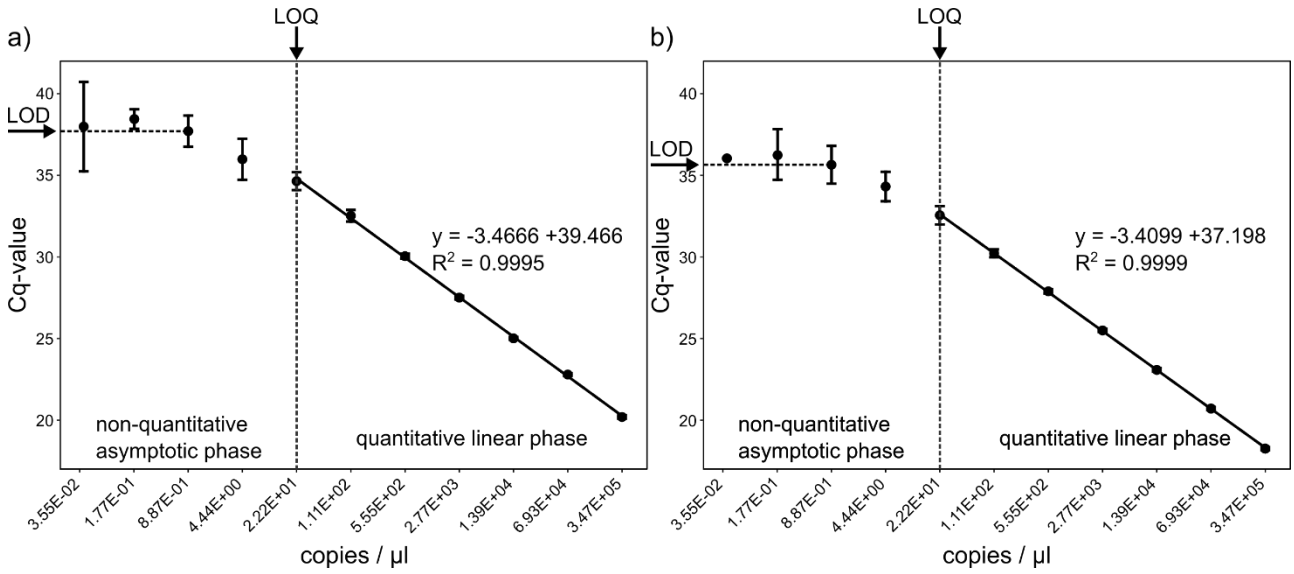


Figure S2. Standard curves for a) *B. dendrobatidis* and b) *T. bryosalmonae*, used for defining the limit of detection (LOD) and limit of quantification (LOQ).

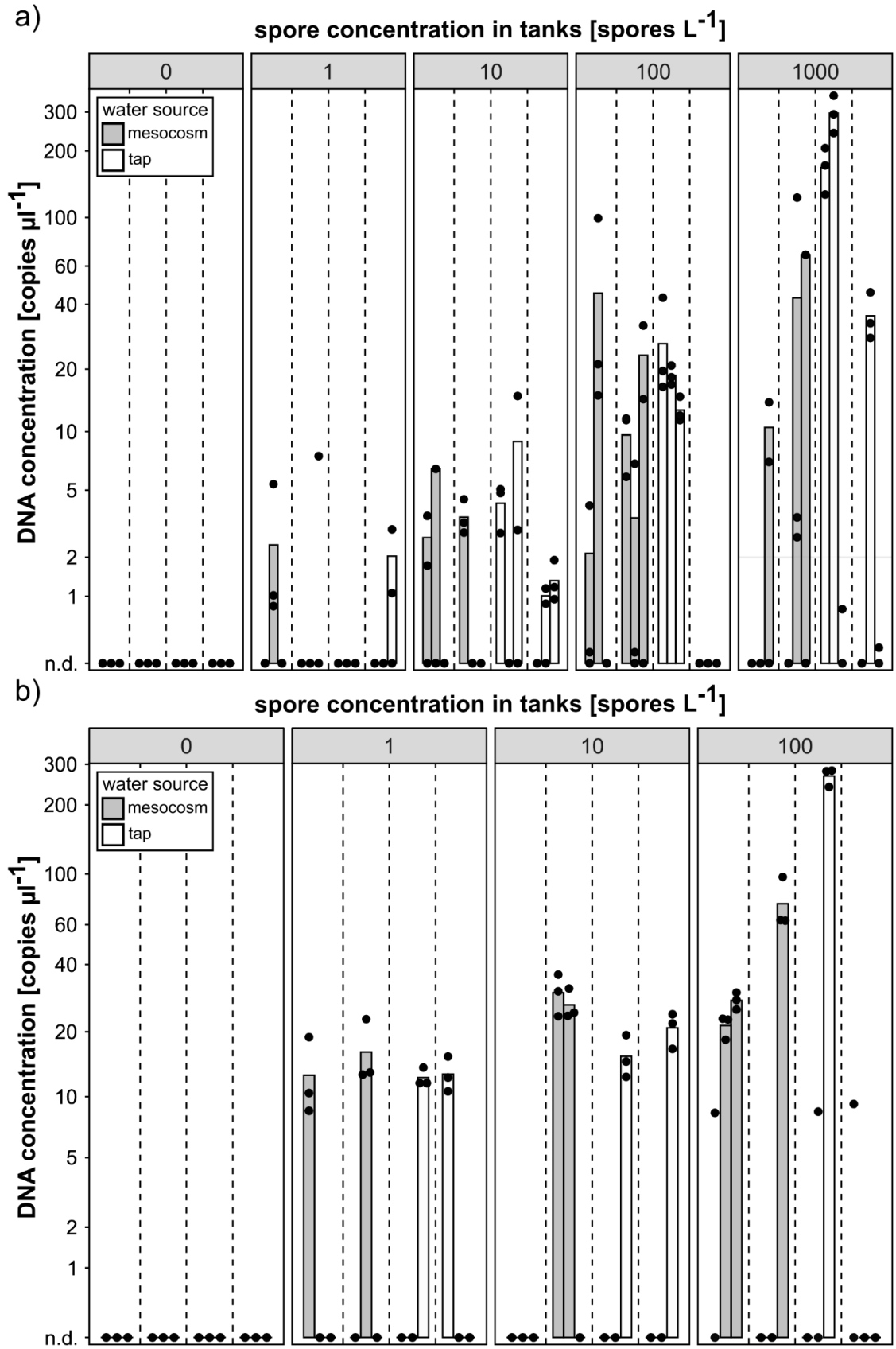


Figure S3. Mean sample DNA concentrations (bars) and replicate DNA concentrations (points) in copies μl^{-1} for a) *B. dendrobatidis* and b) *T. bryosalmonae*. The panels distinguish between different spore concentrations in tanks and dashed lines between samples belonging to different tanks. The points are jittered to better visualise the number of non-detections (n.d.).

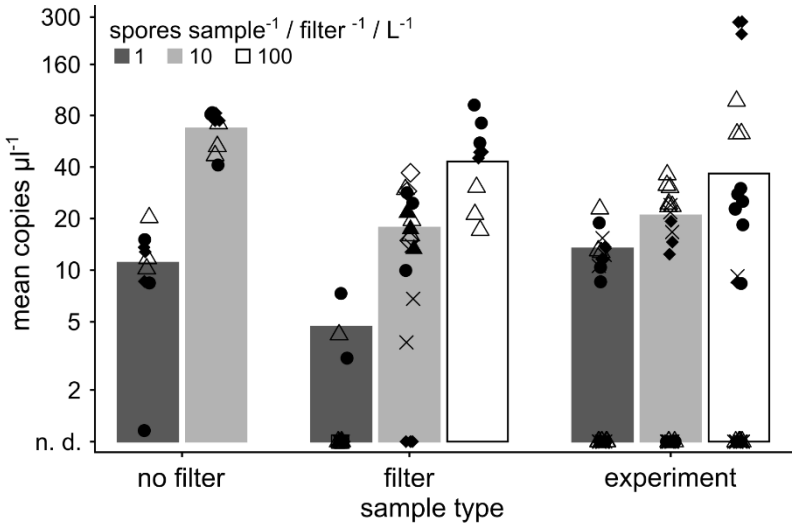


Figure S4. qPCR replicate concentrations of *T. bryosalmonae* DNA in copies μl^{-1} of no filter samples (n = 3 for each spores sample $^{-1}$ treatment), pipette-spiked samples (n = 6 each for treatments of five and 50 spores filter^{-1} and n = 3 for the 500 spores filter^{-1} treatment) and water samples of the experiment (n = 12 for each spore concentration [spores L^{-1}]). Point shapes distinguish between qPCR replicates of different samples for the “no filter” and “filter” sample type categories and samples originating from different tanks for the “experiment” sample type. Non-detections are designated as n. d..

Table S1. Dilution series of Gblocks fragment for absolute quantification and determination of LOD (limit of detection. Standards 5 to 11 were run in 30, and standards 12 to 15 in 40 replicates. Mean Cq-values and detection rates are calculated from all replicates.

				B. dendrobatidis		T. bryosalmonae	
Standard	Dilution	copies / µl	nr. of replicates	mean Cq-values ± SD	% detection	mean Cq-values ± SD	% detection
5	5^-5	3.47E+05	30	20.19 ± 0.11	100	18.25 ± 0.09	100
6	5^-6	6.93E+04	30	22.79 ± 0.08	100	20.71 ± 0.09	100
7	5^-7	1.39E+04	30	25.01 ± 0.08	100	23.08 ± 0.11	100
8	5^-8	2.77E+03	30	27.51 ± 0.11	100	25.50 ± 0.09	100
9	5^-9	5.55E+02	30	30.04 ± 0.15	100	27.89 ± 0.13	100
10	5^-10	1.11E+02	30	32.52 ± 0.35	100	30.22 ± 0.24	100
11	5^-11	2.22E+01	30	34.64 ± 0.55	100	32.55 ± 0.56	100
12	5^-12	4.44E+00	40	35.98 ± 1.26	100	34.31 ± 0.90	100
13	5^-13	8.87E-01	40	37.71 ± 0.96	50	35.65 ± 1.16	50
14	5^-14	1.77E-01	40	38.45 ± 0.60	17.5	36.24 ± 1.51	17.5
15	5^-15	3.55E-02	40	37.98 ± 2.74	7.5	36.04 ± 0 *	2.5

* only one positive replicate

Table S2. Summary of results of the two experiments with *B. dendrobatidis* and *T. bryosalmonae*.

spores L^{-1}	Block no.	water source					
		tap			mesocosm		
		no. positive samples[a]	Cq-value[b]	concentration [copies μl^{-1}][b]	no. positive samples[a]	Cq-value[b]	concentration [copies μl^{-1}][b]
B. dendrobatidis							
1	1	0	n.d.[c]	n.d.[c]	1	37.35	2.41
	2	1	37.25	2.03	1	35.49	7.54
10	1	2	35.94	6.59	2	36.27	4.58
	2	1	37.8	1.18	1	36.46	3.54
100	1	3	34.38	19.26	2	35.94	23.62
	2	0	n.d.[c]	n.d.[c]	3	35.78	12.12
1000	1	2	33.36	155.19	1	35.14	10.49
	2	1	36.71	17.81	2	33.77	55.30
T. bryosalmonae							
1	1	1	34.91	12.31	1	35.24	12.61
	2	1	34.83	12.77	1	34.15	16.16
10	1	1	34.19	15.45	0	NA	NA
	2	1	33.28	20.84	2	32.51	28.22
100	1	1	32.57	137.72	2	34.17	19.12
	2	0[d]	36.19	9.23	1	30.54	74.11

[a] number of samples positive and above LOD for parasite DNA per tank

[b] mean per tank

[c] non-detection

[d] tank had DNA concentrations below LOD which were considered negative

Table S3. List of occupancy models and model fit test results of the posterior Predictive Loss (PPLC) and the Watanabe-Akaike Information criterions (WAIC) computed with the eDNAOccupancy R package (Dorazio & Erickson 2018). Dilution = spore concentration in tank, water = water source, order = order of sampling, block = sample from block 1 or 2, delta Cq = measure of qPCR inhibition.

B. dendrobatidis	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC	
(Ψ(.)θ(.)p(.))	40.745	0.865885	0.91584	0.529530	0.664563	0.00000	0.000000	
(Ψ(.)θ(dilution)p(.))	41.3355	0.883248	0.91295		0.660607	0.59050	0.017364	
(Ψ(.)θ(water)p(.))	40.9961	0.874343	0.92104		0.662885	0.25110	0.008459	
(Ψ(.)θ(order)p(.))	41.447	0.883521	0.92534		0.661069	0.70200	0.017636	
(Ψ(.)θ(block)p(.))	41.0489	0.876883	0.91165		0.662618	0.30390	0.010999	
(Ψ(.)θ(dilution+water)p(.))	41.4716	0.888659	0.91778		0.661506	0.72660	0.022775	
(Ψ(.)θ(dilution+water+order+block)p(.))	43.0594	0.945171	0.92531		0.651639	2.31440	0.079287	
(Ψ(.)θ(dilution+water+order)p(.))	42.2156	0.915201	0.92713		0.658577	1.47060	0.049316	
(Ψ(dilution)θ(.)p(.))	40.7297	0.867027		0.531050	0.663833	**-0.01530**	0.001142	*
(Ψ(water)θ(.)p(.))	40.6527	0.863886		0.525938	0.664564	**-0.09230**	**-0.001999**	**
(Ψ(dilution+water)θ(.)p(.))	40.8686	0.863541		0.508428	0.662324	0.12360	**-0.002343**	*
(Ψ(.)θ(.)p(order))	41.4811	0.896646	0.91330	0.533692		0.73610	0.030762	
(Ψ(.)θ(.)p(block))	41.7101	0.901570	0.91524	0.540272		0.96510	0.035686	
(Ψ(order)θ(.)p(.))	40.8337	0.867934		0.532947	0.664104	0.08870	0.002049	
(Ψ(block)θ(.)p(.))	40.5933	0.862572		0.528000	0.664912	**-0.15170**	**-0.003313**	**
(Ψ(.)θ(.)p(dilution+water))	35.3921	0.789174	0.90824	0.543114		**-5.35290**	**-0.076710**	***
(Ψ(.)θ(.)p(dilution))	39.4818	0.866406	0.91807	0.537746		**-1.26320**	0.000521	*
(Ψ(.)θ(.)p(water))	36.7972	0.805515	0.90783	0.539331		**-3.94780**	**-0.060369**	***
(Ψ(water)θ(.)p(dilution+water))	35.4397	0.788318		0.551458		**-5.30530**	**-0.077566**	***
(Ψ(.)θ(.)p(deltaCq))	40.9544	0.888974	0.91544	0.529587		0.20940	0.023089	
(Ψ(.)θ(deltaCq)p(.))	42.4465	0.903488	0.92594		0.652794	1.70150	0.037603	

T. bryosalmonae	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC	
(Ψ(.)θ(.)p(.))	2.9158	0.128510	0.910035	0.355526	0.955198	0.00000	0.00000	
(Ψ(.)θ(dilution)p(.))	2.9022	0.128049	0.905498		0.955699	**-0.01360**	**-0.00046**	**
(Ψ(.)θ(water)p(.))	2.9007	0.130307	0.906495		0.954823	**-0.01510**	0.00180	
(Ψ(.)θ(dilution+water)p(.))	2.9412	0.129845	0.901019		0.955017	0.02540	0.00134	
(Ψ(.)θ(order)p(.))	2.9561	0.129219	0.910771		0.954536	0.04030	0.00071	
(Ψ(.)θ(block)p(.))	2.9393	0.132541	0.906018		0.953866	0.02350	0.00403	
(Ψ(.)θ(dilution+water+block)p(.))	2.9719	0.131860	0.900802		0.953907	0.05610	0.00335	
(Ψ(dilution+water+block+order)θ(.)p(.))	2.921	0.130122		0.360263	0.954923	0.00520	0.00161	
(Ψ(dilution+water+block)θ(.)p(.))	2.9564	0.130055		0.348725	0.954796	0.04060	0.00155	
(Ψ(dilution+water+order)θ(.)p(.))	2.9366	0.132215		0.359947	0.954629	0.02080	0.00371	
(Ψ(dilution+water)θ(.)p(.))	2.9189	0.129737		0.358618	0.955639	0.00310	0.00123	
(Ψ(dilution)θ(.)p(.))	2.9457	0.130033		0.358381	0.954590	0.02990	0.00152	
(Ψ(water)θ(.)p(.))	2.9471	0.130125		0.357962	0.954304	0.03130	0.00162	
(Ψ(.)θ(.)p(dilution+water+block+order))	1.0022	0.048307	0.910042	0.357671		**-1.91360**	**-0.08020**	**
(Ψ(.)θ(.)p(dilution+water+block))	0.8939	0.040070	0.908960	0.358217		**-2.02190**	**-0.08844**	**
(Ψ(.)θ(.)p(dilution+water))	1.4099	0.054594	0.909885	0.359820		**-1.50590**	**-0.07392**	**
(Ψ(.)θ(.)p(dilution))	2.4458	0.094344	0.909261	0.360003		**-0.47000**	**-0.03417**	**
(Ψ(.)θ(.)p(water))	3.2683	0.134364	0.908365	0.359895		0.35250	0.00585	
(Ψ(.)θ(.)p(dilution+order))	2.4267	0.100685	0.909349	0.357605		**-0.48910**	**-0.02783**	**
(Ψ(.)θ(.)p(dilution+block))	1.4162	0.054181	0.909545	0.359820		**-1.49960**	**-0.07433**	**
(Ψ(.)θ(.)p(deltaCq))	3.3188	0.150064	0.908837	0.359913		0.40300	0.02155	
(Ψ(.)θ(dilution)p(dilution+water))	1.4035	0.054593	0.904206			**-1.51230**	**-0.07392**	**
(Ψ(.)θ(.)p(block))	3.0504	0.114911	0.908541	0.359784		0.13460	**-0.01360**	
(Ψ(.)θ(.)p(order))	3.3075	0.147691	0.909173	0.359605		0.39170	0.01918	

Table S4. Results of *T. bryosalmonae* spike consistency tests for no-filter and pipette-spiked filter samples

spores sample^{-1} / filter $^{-1}$	no. positive replicates[a]	Cq-value[b]	concentration [copies µl^{-1}][b]
no filter samples			
1	3	35.98	8.22
1	3	34.59	14.08
1	3	34.82	11.68
10	3	32.31	68.46
10	3	32.52	57.04
10	3	32.06	77.21
filter samples			
1	0[c]	36.71	4.21
1	0	n.d.[d]	n.d.[d]
1	0	n.d.[d]	n.d.[d]
1	1	36.13	5.19
1	0	n.d.[d]	n.d.[d]
1	0	n.d.[d]	n.d.[d]
10	3	34.36	21.68
10	3	34.09	26.96
10	1	35.94	8.17
10	3	34.08	20.93
10	0	n.d.[d]	n.d.[d]
10	3	34.24	17.47
100	3	32.55	73.18
100	3	34.27	22.93
100	3	33.15	47.65

[a] number of replicates positive and above LOD for parasite DNA per sample

[b] mean per sample

[c] tank had DNA concentrations below LOD which were considered negative

[d] non-detection

Table S5. Data of water, filter and no filter samples spiked with *T. bryosalmonae* spores. Column descriptions can be found below the table. This data was used to create Figure 3.

water	dilution	spore_nr	block	sample	replicate	order	type	meanCq	STDCq	meanconc	STDconc	delta_Cq	rep_delta_Cq
meso	0	0	1	NA	1	NA	control	0	0	0	0	0.756004	0.80049
meso	0	0	1	1	1	NA	control	0	0	0	0	0.900187	1.02049
meso	0	0	1	2	1	NA	control	0	0	0	0	0.835433	1.19049
meso	0	0	1	3	1	NA	control	0	0	0	0	1.156808	1.12049
tap	0	0	1	NA	1	NA	control	0	0	0	0	0.811314	0.50049
tap	0	0	1	1	1	NA	control	0	0	0	0	0.455609	0.93049
tap	0	0	1	2	1	NA	control	0	0	0	0	1.088993	1.14049
tap	0	0	1	3	1	NA	control	0	0	0	0	0.733963	0.68049
meso	0	0	2	NA	1	NA	control	0	0	0	0	0.335254	-0.16951
meso	0	0	2	1	1	NA	control	0	0	0	0	0.563771	0.44049
meso	0	0	2	2	1	NA	control	0	0	0	0	0.502162	0.90049
meso	0	0	2	3	1	NA	control	0	0	0	0	0.53267	0.61049
tap	0	0	2	NA	1	NA	control	0	0	0	0	0.929526	0.78049
tap	0	0	2	1	1	NA	control	0	0	0	0	0.495797	0.47049
tap	0	0	2	2	1	NA	control	0	0	0	0	0.616373	0.27049
tap	0	0	2	3	1	NA	control	0	0	0	0	0.617312	0.49049
meso	1	5	1	NA	1	4	control	0	0	0	0	0.237891	-0.28951
meso	1	5	1	1	1	4	sample	35.244843	1.59886	12.610748	5.490121	0.794237	0.62049
meso	1	5	1	2	1	4	sample	0	0	0	0	0.620548	0.75049
meso	1	5	1	3	1	4	sample	0	0	0	0	1.361206	1.46049
tap	1	5	1	NA	1	6	control	0	0	0	0	-0.0366	0.05049
tap	1	5	1	1	1	6	sample	0	0	0	0	0.380419	0.66049
tap	1	5	1	2	1	6	sample	0	0	0	0	0.339705	0.17049
tap	1	5	1	3	1	6	sample	34.907527	0.36356	12.313675	1.237728	0.717349	0.92049
meso	1	5	2	NA	1	5	control	0	0	0	0	0.240917	0.47049
meso	1	5	2	1	1	5	sample	0	0	0	0	0.952675	1.05049
meso	1	5	2	2	1	5	sample	34.152595	1.00692	16.161156	5.77933	0.947172	0.95049
meso	1	5	2	3	1	5	sample	0	0	0	0	1.620817	1.68049
tap	1	5	2	NA	1	1	control	0	0	0	0	0.694131	0.44049
tap	1	5	2	1	1	1	sample	34.832239	0.69492	12.767666	2.42481	0.519166	0.46049
tap	1	5	2	2	1	1	sample	0	0	0	0	0.756964	1.14049
tap	1	5	2	3	1	1	sample	0	0	0	0	0.831339	0.80049
meso	10	50	1	NA	1	2	control	0	0	0	0	0.482365	0.56049
meso	10	50	1	1	1	2	sample	0	0	0	0	0.744676	0.87049
meso	10	50	1	2	1	2	sample	0	0	0	0	0.487177	0.53049
meso	10	50	1	3	1	2	sample	0	0	0	0	0.502245	0.55049
tap	10	50	1	NA	1	3	control	0	0	0	0	0.352662	-0.04951
tap	10	50	1	1	1	3	sample	0	0	0	0	0.529394	0.53049
tap	10	50	1	2	1	3	sample	0	0	0	0	0.351624	0.44049
tap	10	50	1	3	1	3	sample	34.193682	0.72118	15.445685	3.559307	0.584282	0.59049
meso	10	50	2	NA	1	2	control	0	0	0	0	0.41008	0.24049
meso	10	50	2	1	1	2	sample	32.375603	0.51091	29.988593	6.341058	3.695338	3.39049
meso	10	50	2	2	1	2	sample	32.651687	0.37005	26.44152	4.235068	0.856654	0.85049
meso	10	50	2	3	1	2	sample	0	0	0	0	0.422124	0.44049
tap	10	50	2	NA	1	4	control	0	0	0	0	0.441649	-0.44951
tap	10	50	2	1	1	4	sample	0	0	0	0	0.735196	0.86049
tap	10	50	2	2	1	4	sample	0	0	0	0	0.634465	0.74049
tap	10	50	2	3	1	4	sample	33.278746	0.52415	20.840038	3.77836	0.939601	0.84049

Table S5. continued.

water	dilution	spore_nr	block	sample	replicate	order	type	meanCq	STDCq	meanconc	STDconc	delta_Cq	rep_delta_Cq
meso	100	500	1	NA	1	5	control	0	0	0	0	0.221777	0.24049
meso	100	500	1	1	1	5	sample	36.804131	0	8.3711968	0	0.16789	0.54049
meso	100	500	1	2	1	5	sample	33.188154	0.33928	21.339646	2.569103	0.66835	0.69049
meso	100	500	1	3	1	5	sample	32.524471	0.21041	27.663113	2.420939	0.391698	0.49049
tap	100	500	1	NA	1	1	control	0	0	0	0	0.270602	0.16049
tap	100	500	1	1	1	1	sample	0	0	0	0	0.170327	0.15049
tap	100	500	1	2	1	1	sample	36.711782	0	8.4809129	0	0.277885	0.06049
tap	100	500	1	3	1	1	sample	28.427866	0.13486	266.95545	23.98825	0.290432	-0.05951
meso	100	500	2	NA	1	6	control	0	0	0	0	0.409184	-0.02951
meso	100	500	2	1	1	6	sample	0	0	0	0	0.666866	0.52049
meso	100	500	2	2	1	6	sample	0	0	0	0	0.642604	0.77049
meso	100	500	2	3	1	6	sample	30.537174	0.45311	74.110256	19.87227	0.811078	1.00049
tap	100	500	2	NA	1	3	control	0	0	0	0	0.610514	0.70049
tap	100	500	2	1	1	3	sample	36.185111	0	9.2329877	0	0.266508	0.64049
tap	100	500	2	2	1	3	sample	0	0	0	0	0.715388	0.68049
tap	100	500	2	3	1	3	sample	0	0	0	0	0.921441	1.12049
NA	NA	5	NA	NA	1	NA	filter	36.711338	0	4.2134738	0	NA	NA
NA	NA	5	NA	NA	1	NA	filter	0	0	0	0	NA	NA
NA	NA	5	NA	NA	1	NA	filter	0	0	0	0	NA	NA
NA	NA	5	NA	NA	1	NA	filter	0	0	0	0	NA	NA
NA	NA	5	NA	NA	1	NA	filter	0	0	0	0	NA	NA
NA	NA	5	NA	NA	1	NA	filter	36.125643	0.91122	5.1943636	3.005691	NA	NA
NA	NA	50	NA	NA	1	NA	filter	34.356223	0.4625	21.682093	7.067827	NA	NA
NA	NA	50	NA	NA	1	NA	filter	34.085768	0.68838	26.961838	11.16233	NA	NA
NA	NA	50	NA	NA	1	NA	filter	35.943537	0.95453	8.1684196	5.187928	NA	NA
NA	NA	50	NA	NA	1	NA	filter	34.082477	0.82511	20.929563	9.677661	NA	NA
NA	NA	50	NA	NA	1	NA	filter	0	0	0	0	NA	NA
NA	NA	50	NA	NA	1	NA	filter	34.235365	0.35207	17.465819	4.135386	NA	NA
NA	NA	500	NA	NA	1	NA	filter	32.552081	0.37384	73.183544	18.36124	NA	NA
NA	NA	500	NA	NA	1	NA	filter	34.267074	0.43067	22.931216	6.892384	NA	NA
NA	NA	500	NA	NA	1	NA	filter	33.151591	0.06954	47.651931	2.225898	NA	NA
NA	NA	5	NA	NA	1	NA	no filter	35.975578	1.999	8.2196397	6.958514	NA	NA
NA	NA	5	NA	NA	1	NA	no filter	34.590351	0.53275	14.078438	5.472001	NA	NA
NA	NA	5	NA	NA	1	NA	no filter	34.823795	0.36422	11.679989	2.681381	NA	NA
NA	NA	50	NA	NA	1	NA	no filter	32.305147	0.57295	68.461819	23.69255	NA	NA
NA	NA	50	NA	NA	1	NA	no filter	32.520446	0.31678	57.036154	13.03257	NA	NA
NA	NA	50	NA	NA	1	NA	no filter	32.064408	0.08391	77.205831	4.608556	NA	NA

Table legend:

water	=	water source (mesocosm or tap)
dilution	=	spore concentration (spores L^{-1}) in tank
spore_nr	=	spore concentration (spores L^{-1})*L
block	=	block number
sample	=	sample number (one control and 3 samples per tank)
order	=	order of filtration (nth tank to be filtered during the experiment)
type	=	negative control, experimental sample, lab-spiked filter or no-filter sample
meanCq	=	mean Cq-value of positive replicates of the sample
STDCq	=	standard deviation of Cq-value per sample
meanconc	=	mean DNA concentration (copies ul^{-1}) of positive replicates of the sample
STDconc	=	standard deviation of concentration per sample
delta_Cq	=	Cq deviation from IPC standard
rep_delta_Cq	=	Cq deviation of the qPCR replicate from IPC standard

References:

Dorazio RM, Erickson RA (2018) Ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380.

Chapter 2

Parasite DNA detection in water samples enhances crayfish plague (*Aphanomyces astaci*) monitoring in asymptomatic carrier populations

Natalie Sieber[1,2], Hanna Hartikainen[1,2,3], Raphael Krieg[4], Armin Zenker[4], Christoph Vorburger[1,2]

[1] Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

[2] ETH Zürich, Institute of Integrative Biology (IBZ), 8092 Zürich, Switzerland

[3] University of Nottingham, School of Life Sciences, Nottingham, UK

[4] University of Applied Sciences and Arts Northwestern Switzerland, 4132 Muttenz, Switzerland

Submitted to Biological Invasions

Abstract

Invasive species can facilitate the spread of pathogens by providing asymptomatic reservoirs of hosts, where pathogen spillover to native host populations may lead to disease outbreaks. Invasive North American crayfish act as carriers of the crayfish plague (*Aphanomyces astaci*), posing a major disease threat to European freshwater crayfish populations. Effective *A. astaci* disease management requires comprehensive monitoring, however, pathogen detection in carrier populations with low infection prevalence and intensities can be challenging. We compared tissue and water sample based detection success of *A. astaci* by simultaneously collecting and analysing crayfish cuticle samples and water samples of invasive crayfish populations. Using quantitative real-time PCR *A. astaci* was detected with signal strengths above the limit of detection (LOD) in 13 of 23 invasive crayfish populations, but only in four populations with both methods. If weak signals below LOD from water samples are also considered, positive sites increased to 17, but still only seven sites with detection by both methods. The likely reason for the discrepancies is the low *A. astaci* prevalence in resistant American crayfish and, accordingly, low spore concentrations that limit detection reliability. Consistency may be improved by timing the surveys with seasonal periods of high *A. astaci* abundance and by increased water sampling effort. Considering the low effort required for environmental DNA monitoring compared to crayfish trapping and tissue sampling, a combination of both would facilitate regularly updated monitoring campaigns and provide a more comprehensive picture of *A. astaci* distribution.

Keywords: *Aphanomyces astaci*, invasive crayfish, environmental DNA, pathogen monitoring

Introduction

Invasive species can disrupt the structure and functioning of communities and ecosystems, and threaten the survival of endangered species (Strayer 2010). Co-dispersal of parasites with invasive host species often compounds the harmful effects on local biodiversity, especially when the introduced parasite can also infect resident biota (Dunn & Hatcher 2015). The spread of a species carrying parasites into new territory can lead to novel combinations of parasites and hosts, i.e. to a spillover event, where the parasite acquires a new host species in its invasive range (Strauss et al. 2012). In their introduced range, invasive parasites can mediate the competition between species (Price et al. 1988, Dunn & Hatcher 2015). If the new native host is more susceptible to the parasite than its original, non-native host, the non-native host can acquire a competitive advantage, which promotes its spread and its chances of becoming invasive (Strauss et al. 2012). Invasive species and their parasites can therefore become serious threats to highly susceptible native species, since the invasive species can act as an asymptomatic carrier and reservoir species for the parasite. Such reservoir species can be crucial for the persistence of an invasive parasite (Reynolds 1988).

The oomycete *Aphanomyces astaci* is the causative agent of crayfish plague, the most serious disease threatening European native freshwater crayfish species (Holdich et al. 2009). It is therefore listed among the 100 worst invasive species worldwide (Lowe et al. 2000). Native European crayfish are highly susceptible to the disease, which is transmitted by free-swimming zoospores, and local population extinction have been documented in a matter of weeks after contracting the pathogen (Unestam & Weiss 1970, Alderman et al. 1987). Originating from North America, *A. astaci* has a long history of co-evolution with North American crayfish species, such as the signal crayfish (*Pacifastacus leniusculus*), which appear to be asymptomatic carriers of the pathogen in their introduced ranges in Europe (Holdich et al. 2009). Infection in crayfish usually happens through lesions in the epicuticle, the outermost layer of the exoskeleton (Unestam & Weiss 1970). Once infected, growth of *A. astaci* hyphae is stopped or slowed by melanisation in *P. leniusculus*, while the melanisation response of the European noble crayfish (*Astacus astacus*) is too slow to prevent the parasite from spreading (Nyhlén & Unestam 1980, Cerenius et al. 2003). The widespread presence of invasive North American crayfish species in Europe, acting as disease carrier and reservoir species, poses severe infection risks to native crayfish populations (Holdich et al. 2009, Kouba et al. 2014). Crayfish transported in the ballast water of trans-Atlantic ships are suspected as the source of initial *A. astaci* invasions (Holdich

2003). The first outbreak was recorded in Italy in 1859 and several outbreaks were observed throughout Europe thereafter (Alderman 1996). The intentional release of *P. leniusculus* in Sweden, to compensate for the dwindling populations of native *A. astacus*, further promoted the spread of the crayfish plague (Bohman et al. 2006). Today, invasive North American crayfish and *A. astaci* are found in most European countries (Kouba et al. 2014).

In Switzerland, three North American crayfish species, *P. leniusculus*, spiny-cheek (*Faxonius limosus*) and red swamp crayfish (*Procambarus clarkii*) have successfully colonised large waterways and lakes, while populations of the native species *A. astacus*, white-clawed (*Austropotamobius pallipes*) and stone crayfish (*Austropotamobius torrentium*) still persist in isolated waterbodies or smaller, hard to reach streams (Stucki & Zaugg 2005). To preserve the remaining populations and allow their recovery, management plans have been devised (Stucki & Zaugg 2011, Elmiger et al. 2018). For the effective implementation of such plans, close surveillance of native and invasive crayfish populations and their disease status is crucial. The advancement of molecular methodologies has enabled the development of fast and reliable *A. astaci* detection using PCR (Oidtmann et al. 2002, 2004, 2006, Hochwimmer et al. 2009) and quantitative real-time PCR (qPCR) assays (Vrålstad et al. 2009) of infected crayfish tissue. Molecular diagnostics on crayfish tissue samples have therefore become the default testing method for crayfish plague (Kozubíková et al. 2009, Vrålstad et al. 2011, Kokko et al. 2012, Schrimpf et al. 2012). Soft cuticle from the abdomen and the tail fans of invasive crayfish species have shown highest *A. astaci* detection rates and sampling of both cuticle types increases detection success (Oidtmann et al. 2006, Vrålstad et al. 2011). However, acquisition of crayfish tissue for testing is laborious since crayfish need to be captured in high numbers for reliable results of infection status, especially when *A. astaci* prevalence in the population is low (Schrimpf et al. 2013). Therefore, an alternative method involving the detection of *A. astaci* in water samples, based on the concept of environmental DNA (eDNA; Bass et al. 2015, Thomsen & Willerslev 2015), has been developed and experimentally tested with water spiked with *A. astaci* zoospores (Strand et al. 2011), with ambient water of infected *P. leniusculus* (Strand et al. 2012), and successfully applied in lakes with *A. astaci* positive *P. leniusculus* populations (Strand et al. 2014). In field surveys, methods for detection of *A. astaci* in water have performed equally well, or better, in detecting infected sites, than crayfish trapping and tissue extraction methods (Strand et al. 2014, Wittwer et al. 2018, 2019). Being less cost- and labour-intensive than conventional trapping methods with subsequent examination of single

crayfish individuals, the eDNA-based method can greatly facilitate a regularly updated *A. astaci* monitoring scheme. For this, rigorous validation is required to assess the efficacy and reliability of such an eDNA-based detection method in comparison to more established methods.

In this study we assessed the performance of an eDNA-based method in detecting *A. astaci* in water in comparison to crayfish trapping combined with molecular detection in crayfish tissue. The main aim was to evaluate the capacity of eDNA-based method to detect the crayfish plague agent associated with invasive, asymptomatic crayfish populations with low infection intensity levels. To achieve this, we firstly examined the degree of association between *A. astaci* detection results of both methods. Secondly, we investigated sources of variation in detection for both methods, including host species identity, crayfish size and sex, *A. astaci* prevalence and number of infected individuals among captured crayfish and agent levels in crayfish. Third, to confirm the functionality of both methods in a situation with high *A. astaci* concentrations in crayfish and water, we sampled *A. astacus* and water samples from an active crayfish plague outbreak site. Detection rates and DNA concentrations were compared to those from asymptomatic invasive crayfish populations. Lastly, we discuss the implications of our findings for crayfish plague monitoring.

Methods

Site selection and crayfish sampling

Sampling sites were chosen using prior knowledge of invasive crayfish species occurrence in the Canton of Zürich (ZH), Switzerland (n = 21 sites, Table 1). Three additional sites were sampled in the Cantons Aargau (AG), St. Gallen (SG) and Zug (ZG). Sampling sites comprised different types of waterbodies, ranging from small brooks to large rivers and lakes. Sampling was conducted from May to September 2017, except for the river Glatt, which was sampled in September 2018 when a crayfish plague outbreak was discovered in its native *A. astacus* population. Nine sites were inhabited by *P. leniusculus*, six by *F. limosus*, six by *P. clarkii* and a mixed population of *P. leniusculus* and *F. limosus* was found at one site (Table 1).

Table 1. Details of surveyed sites: waterbody type, WGS84 coordinates, sampling date, number of eDNA samples and total volume of water filtered in litres, the crayfish species present at the site, the number of crayfish analysed and number of crayfish per *A. astaci* agent level (A0 – A7), the *A. astaci* prevalence of the analysed crayfish (number of infected crayfish / number of analysed crayfish) and *A. astaci* eDNA results (n. d. = no detection, below LOD = *A. astaci* DNA concentrations below limit of detection, above LOD = *A. astaci* DNA concentrations above limit of detection).

site name	type	WGS84		sampling date	nr. eDNA samples	total volume filtered [L]	crayfish species	nr. of crayfish	agent levels						*A. astaci* prevalence [%]	*A. astaci* eDNA
		E	N						A_0	A_1	A_2	A_3	A_4	A_5		
Bienzerliweiher	pond	8.40307	47.42178	03.08.2017	3	15	*F. limosus*	1		1					100	below LOD
Weiher Gheid	pond	8.44392	47.44807	20.09.2017	6	8	*P. clarkii*	21	21						0	n. d.
Rhein	river	8.59458	47.60261	05.07.2017	3	15	*F. limosus*	1	1						0	n. d.
Rhein	river	8.43490	47.56665	08.08.2017	4	15.5	*F. limosus*	11	11						0	below LOD
Zugersee	lake	8.50408	47.17298	06.09.2017	3	15	*F. limosus*	20	5	7	6	2			75	above LOD
Landbach	stream	8.47226	47.58444	26.06.2017	3	15	*F. limosus*	20	1	5	7	7			80	n. d.
Mattenbach A	stream	8.77525	47.47686	04.08.2017	3	15	*P. leniusculus*	3	3						0	above LOD
Mattenbach B	stream	8.73559	47.49367	14.08.2017	3	15	*P. leniusculus*	34	34						0	below LOD
Raffoltersee	pond	8.78623	47.62108	05.07.2017	3	15	*P. leniusculus*	39	34	5					5.1	n. d.
Chuesenbach	stream	8.59350	47.32651	19.07.2017	3	15	*P. leniusculus*	21	13	7	1				23.8	below LOD
Riedbach	stream	8.46253	47.48610	16.05.2017	3	15	*P. leniusculus*	21	3	12	4	2			57.1	above LOD
Furtbach A	stream	8.49086	47.43778	20.06.2017	6	15	*P. clarkii*	11	11						0	n. d.
Furtbach B	stream	8.46613	47.44510	20.06.2017	3	15	*P. clarkii*	3	3						0	below LOD
Katzensee	lake	8.49285	47.43134	06.07.2017	6	12	*P. clarkii*	29	27	2					0	n. d.
Waldbach	stream	8.74840	47.48294	14.08.2017	6	10	*P. leniusculus*	27	26	1					0	below LOD
Rumensee	pond	8.59096	47.32891	18.07.2017	6	10.5	*P. clarkii*	10	8	1	1				20	n. d.
Schübelweiher	pond	8.59399	47.32405	18.07.2017	6	4	*P. clarkii*	13	9	2	2				30.8	n. d.
Limmat A	river	8.40253	47.42181	03.08.2017	3	15	*F. limosus*	22	9	6	5	2			40.9	below LOD
Rhein Tössegg	river	8.55504	47.55189	08.08.2017	3	15	*F. limosus* *P. leniusculus*	26	25	1					3.8	above LOD
Töss	river	8.65234	47.51873	27.09.2017	3	15	*P. leniusculus*	22	22						0	n. d.

Table 1. (cont.)

site name	type	WGS84		sampling date	nr. eDNA samples	total volume filtered [L]	crayfish species	nr. of crayfish	agent levels						*A. astaci* prevalence [%]	*A. astaci* eDNA
		E	N						A_0	A_1	A_2	A_3	A_4	A_5		
Girhaldeweiher	pond	8.69098	47.43137	22.05.2017	6	15	*P. leniusculus*	28	27	1[a]					0	n. d.
Limmat B	river	8.31733	47.45533	30.08.2017	3	15	*P. leniusculus*	22	4	4	9	5			72.7	above LOD
Greifensee	lake	8.69479	47.33219	08.06.2017	3	15	*F. limosus*	26	23	3					11.5	n. d.
Glatt	stream	9.15627	47.43941	28.09.2018	3	15	*A. astacus*	8			1	3		4	100	above LOD

[a]*A. astaci* DNA concentration in tissue was below limit of detection (LOD)

Depending on accessibility and practicability, crayfish were either captured by hand, trapped, or both (Table 1). Hand-capture was conducted at daytime by searching the crayfish underneath stones and in potential burrows. If less than 20 crayfish were caught by hand, five baited traps (Krebskorb Pirat, Engel-Netze GmbH &Co.KG, Bremerhaven, Germany) were distributed at the sampling site and left overnight. The river Töss was trapped over two nights with 10 baited traps each night by A. Gouskov and crayfish from Lake Greifensee were bought from a local fisherman. The River Limmat near Neuenhof AG was trapped with 10 traps for one night and crayfish in Lake Zugersee were captured as bycatch in nets and kindly provided by fisherman P. Reichlin. The captured crayfish were anaesthetised with clove oil (Ghanawi et al. 2019), and frozen at -20°C until tissue extraction.

eDNA sampling

Environmental DNA samples were collected on the same date as the crayfish at each sampling site, except for sites "Greifensee", "Limmat Neuenhof" and "Zugersee". Crayfish and water samples were collected between mid-May and end of September 2017 (Table 1). The water sampling procedure is described in detail and visualised in Fig. 1 in Sieber et al. (in press). In short, a portable peristaltic pump (Alexis peristaltic pump, Proactive Environmental Products LLC, Bradenton FL, USA) was used to pump water through a 47 mm diameter glass fibre filter (Grade GF/B, Whatman, VWR, Dietikon, Switzerland). The tubes containing the eDNA filters were transferred on ice before being stored at -80°C until extraction. Three 5 L water samples were collected per site, except when filters clogged early due to suspended particles in the water. In that case, up to six water samples, i.e. filters, were collected. At each sampling site, 5 L of clean MilliQ water were first filtered through the filtration equipment as a negative control to verify cleanliness of the equipment.

Crayfish tissue and environmental DNA extraction

Carapace length and sex of each crayfish was determined before tissue sampling. We extracted half of the soft abdominal cuticle and three of the five tail fan tips (uropods) from each crayfish (Fig. 1). The cuticle and uropod samples were stored separately at -20°C and DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen AG, Hombrechtikon, Switzerland), following a protocol adapted from Strand et al. 2019. Tissue samples were transferred to 2 ml tubes containing one steel bead (5 mm; Qiagen AG, Hombrechtikon, Switzerland), and frozen at -80°C for at least 10 minutes. Frozen tissue was then crushed

using a TissueLyser II (Qiagen AG, Hombrechtikon, Switzerland) for 30 seconds at full speed. This step was repeated if the tissue was not properly crushed after the first time. After, the tissue was thawed at 56°C and 800 µl of ATL buffer was added and another bead beating step was conducted. To remove foam, the samples were then centrifuged for a minute at maximum speed. We then froze the samples again at -80°C for a minimum of 10 minutes and thawed them at 56°C. Ten µl of proteinase K (20 mg ml^{-1}) was added and mixed, and samples were then incubated at 56°C overnight. The next day, samples were centrifuged for 5 minutes at 12'000 x g, followed by a transfer of 550 µl of supernatant to a new 2 ml tube. We then added 550 µl of AL buffer and mixed the samples thoroughly before incubation at 56°C for 10 minutes. Then, 550 µl of ethanol (96 – 100 %) were added and mixed and 650 µl of the sample was transferred to a DNeasy Mini spin column placed in a 2 ml collection tube. The spin columns were centrifuged at 6000 x g for one minute and the flow-through discarded. This step was repeated until all liquid had been processed. The spin column was then placed into a new collection tube and 500 µl of AW1 buffer were added. The samples were centrifuged at 6000 x g for one minute and the flow-through was discarded. We then added 500 µl of AW2 buffer, followed by centrifugation at 20'000 x g for 3 minutes. The flow-through and collection tube were discarded, and the spin column was transferred to a new 1.5 ml tube. The DNA was eluted with 200 µl of AE buffer, incubation of one minute at room temperature and centrifugation at 6000 x g for one minute. At the beginning of the project, we also used a CTAB-based and a high salt extraction method on a subset of the sampled crayfish before settling on the DNEasy Blood and Tissue kit. The protocols of all extraction protocols can be found in the Supplement (Text S1 – S3).

A dedicated laboratory used only for processing sensitive samples with low DNA content and for pre-PCR work was used for eDNA extractions. Environmental DNA samples were extracted with the DNEasy Power Water kit (Qiagen AG, Hombrechtikon, Switzerland) as described in Sieber et al. (in press). Extraction runs included a no-template extraction control. The extracted DNA samples were stored at -20°C until further analysis.

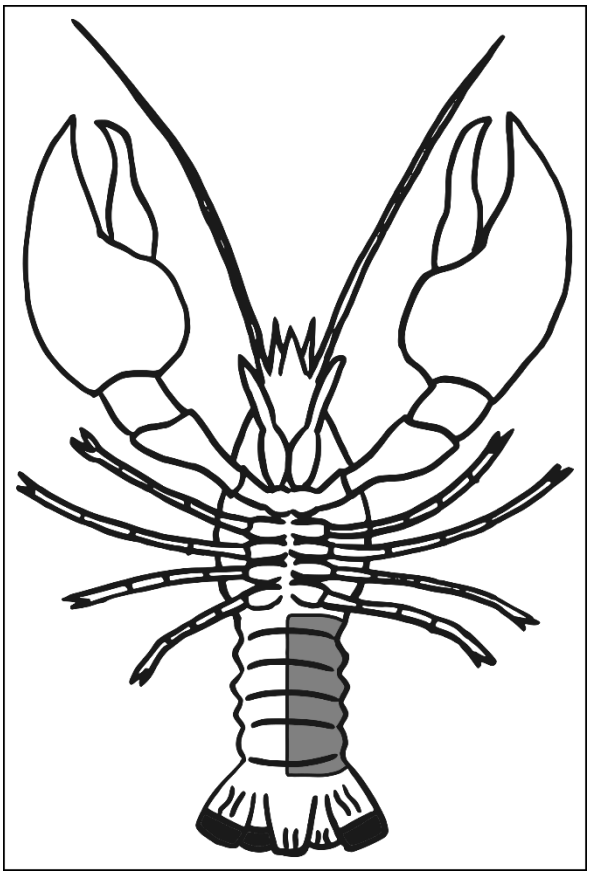


Figure 1. Schematic showing crayfish tissue samples extracted for analysis of *A. astaci*. Cuticle sample shown in grey (comprising half of the soft abdominal cuticle) and uropod sample in black (comprising tips of three uropods). Drawn after Vrålstad et al. (2011).

Real-time quantitative PCR

Both crayfish tissue and eDNA extracts were analysed for *A. astaci* with real-time quantitative PCR (qPCR) on a LightCycler 480 (Roche, Basel, Switzerland) using the same procedures and protocol. A QIAgility pipetting robot (Qiagen AG, Hombrechtikon, Switzerland) was used for setting up triplicate reactions. For quantification of the samples, a five-fold dilution series consisting of eight dilutions of a double-stranded Gblocks fragment (Integrated DNA Technologies) containing the *A. astaci* assay target sequence (see Fig. S1 in the Supplement for sequence information) was included in each qPCR run. The DNA concentrations ranged from 69'335 copies μl^{-1} to 0.9 copies μl^{-1}. A negative PCR control was included in each qPCR run. The assay developed by Vrålstad et al. (2009) with a modified thermal cycling regime to reduce non-specific amplification according to Strand et al. (2011, 2014) was used. Probe and primer concentrations were optimised for Roche 480 Probes Master Mix in 10ul reactions. Reactions contained 5 µl of LightCycler 480 Probes Master buffer (Roche, Basel, Switzerland), forward primer AphAstITS-39F at concentration of 50 nM, reverse primer AphAstITS-97R at 900 nM, the MGB probe AphAstITS-60T (Vrålstad et al. 2009) at 200 nM and 2.5 µl of template DNA. Thermal cycling was initiated by 10 minutes at 95°C to activate the DNA polymerase and denature template DNA,

followed by 50 cycles of 15 seconds at 95°C and 30 seconds at 62°C. At the end, a cooling step of 10 seconds at 40°C was implemented as suggested by the manufacturer of the thermal cycler. A synthetic template not matching any published sequence data was used as internal positive control to test for PCR inhibition of crayfish tissue and eDNA samples (Carraro et al. 2017). The IPC reactions were setup using methods described in Sieber et al. (in press). The IPC was run separately from the *A. astaci* assays in triplicate for each crayfish tissue and eDNA sample.

Limit of detection and limit of quantification

To determine the *A. astaci* assay limit of detection (LOD) and limit of quantification (LOQ) the double-stranded Gblocks fragment (Integrated DNA Technologies) containing the *A. astaci* assay target sequence was diluted five-fold to create a dilution series with 15 dilutions from concentrations of $54.17*10^7$ to 0.09 copies reaction^{-1}. A qPCR run with the *A. astaci* assay was conducted with 30 replicates each of the dilutions 5 to 11, and 40 replicates each of dilutions 12 to 15 (see Table S1 in the Supplement for detailed results). Detection rates were a 100 % in all replicates up to dilution 12. Therefore, the mean cycle value (Cq-value) of positive replicates of dilution 13 with 62.5 % detection success was defined as LOD (Cq-value = 38.844; 2.22 copies reaction^{-1}). This is a more permissive LOD than the frequently used 95% detection threshold (Bustin et al. 2009), which we deemed appropriate for low content DNA samples of pathogens. For comparison, we also calculated the LOD of the *A. astaci* assay at a 95% detection level according to Klymus et al. (2019), which lies at a concentration of 7.76 copies reaction^{-1}. The LOQ was defined as the concentration of the last dilution of the linear range of the standard curve, which was at 11.09 copies reaction^{-1}. The standard curve used to define the LOD and LOQ is visualised in Fig. S2 in the Supplement.

Agent Levels

To semi-quantitatively categorise *A. astaci* loads in crayfish tissue, the PCR forming unit (PFU) value of dilution 13 mentioned above was calculated using most probable number (MPN) estimation (Blodgett 2010), i.e. PFU reaction^{-1} = $2.303*\log10(n*q^{-1})$ were n = total number of qPCR replicates and q = number of negative qPCR replicates. Dilution 13 had 15 out of 40 negative replicates, resulting in PFU reaction^{-1} (dilution 13) = 0.981. This value was used to calculate the PFU values of the remaining dilutions of the series. With the PFU

values and mean DNA concentrations for each dilution of the standard curve, the curve equation $y = -1.56*\ln(x)+39.71$ was calculated (x = PFU reaction^{-1}, y = concentration). This equation was used to categorise the crayfish tissue samples into agent levels A0 – A7 according to Vrålstad et al (2009): A0: no detection (n. d.), A1: PFU < 5, A2: 5 ≤ PFU < 50, A3: 50 ≤ PFU < 10^3, A4: 10^3 ≤ PFU < 10^4, A5: 10^4 ≤ PFU < 10^5, A6: 10^5 ≤ PFU < 10^6, A7: PFU ≥ 10^6.

Data analysis

The LightCycler 480 Software version 1.5.1 (Roche, Basel, Switzerland) was used to prepare the qPCR raw data as described in Sieber et al. 2020. A crayfish tissue or water sample was considered above LOD if the Cq-value was lower than the LOD in at least one of the three replicate qPCR reactions. Detection of *A. astaci* was considered successful in crayfish if at least one of the two tissue types were tested positive for *A. astaci* DNA. The water at a sampling site was considered *A. astaci* positive if parasite DNA was detected in at least one of the water samples collected at this site. Statistical analyses were conducted in R version 3.6.1. (R Core Team 2019). Parasite detection success was compared between crayfish tissue types and between tissue and water samples using McNemar's chi-square tests. Main and interaction effects of crayfish species, gender and size (carapace length) on *A. astaci* detection success in tissue samples were tested with generalised linear mixed effects models (GLMM), and the effects on estimated *A. astaci* DNA concentrations in tissue samples with linear mixed effects models (LMM), both including sampling site as a random factor. *A. astaci* concentration estimates from different tissue types of the same individual were compared using a Wilcoxon signed-rank test with continuity correction. The effects of parasite prevalence (number of infected / total number crayfish), maximum parasite agent level and number of infected crayfish on *A. astaci* detection success in eDNA samples were analysed with GLMMs. Type II Wald chi-square tests were used to test significance of fixed effects. Linear models were used to analyse the correlation between cuticle and uropod tissue types, including species as factor, and to test for effects of parasite prevalence, crayfish agent levels, and mean *A. astaci* concentrations in crayfish tissue on estimated *A. astaci* eDNA concentrations at the same sampling site. For analyses involving *A. astaci* prevalence, we only included sites where three or more crayfish were captured. PCR inhibition in DNA samples was quantified using the difference of the IPC's Cq-values from qPCR reactions containing the DNA extractions and control reactions containing MilliQ water instead of

DNA template. We considered Δ Cq-values ≥ 3 to indicate substantial inhibition. The effect of inhibition on detection success and estimated DNA concentrations was analysed with binomial GLMs and LMs, respectively. Linear models were used to investigate the effect of crayfish size on PCR inhibition. Kruskal-Wallis rank sum tests were used to test differences of inhibition between invasive crayfish species. A Wilcoxon signed rank test with continuity correction was used to compare inhibition between tissue samples of the same crayfish. All tests analysing effects on detection and parasite DNA concentration were conducted once with the complete dataset and once with a subset of samples extracted with the DNEasy Blood and Tissue kit only. This was to ensure that inclusion of samples not extracted with the kit would not drastically change the results. If not stated otherwise, the presented results are from the full dataset.

The R package "eDNAOccupancy" (Dorazio & Erickson 2018) was used for computing hierarchical occupancy models and model selection to test if parasite prevalence in the population, total number of infected crayfish, crayfish agent levels and inhibition scores had an effect on detection probability. Occupancy models were run with 11'000 iterations of the MCMC algorithm. The posterior Predictive Loss (PPLC, Gelfand & Ghosh 1998) and Watanabe-Akaike Information criterions (WAIC, Watanabe 2010) were used for model selection. If adding a covariate or factor did not improve model fit, the covariate was considered not to influence *A. astaci* detection. The equation $1 - (1-\theta)^n \geq 0.95$ was used to determine the number of water samples (n) required for successful detection probability of 95 %, with θ being the probability of detection of *A. astaci* DNA in a water sample.

Results

Detection of *A. astaci* in water and crayfish tissue

Aphanomyces astaci DNA was detected in quantities above LOD in water from five and in tissue samples from twelve out of 23 sites with invasive crayfish (Fig. 2, Table 1). Crayfish tissue sampling was therefore more successful in detecting *A. astaci* in a population than eDNA sampling (McNemar's $\chi^2 = 4$, df = 1, p = 0.046). *A. astaci* was detected in both crayfish tissue and water from four sites. Of the remaining eight sites with detections in tissues, three sites showed a weak signal in water (below LOD) and the other five had no detection in water. On the other hand, the water samples revealed the presence of *A. astaci* (above LOD) in one site where it was not detected in crayfish tissue.

Weak, below-LOD signals of *A. astaci* were obtained from water in additional four sites where none of the crayfish (n = 75) were found infected (Table 1). Six sites were negative for *A. astaci* in both eDNA and tissue samples. Overall, both eDNA and tissue - based detection revealed 17 *A. astaci* positive sites, if below LOD detections of *A. astaci* in water were included. With their inclusion the detection methods agreed on *A. astaci* presence in seven sites, on its absence in six sites, and five sites each revealed detection in either eDNA or in tissue only.

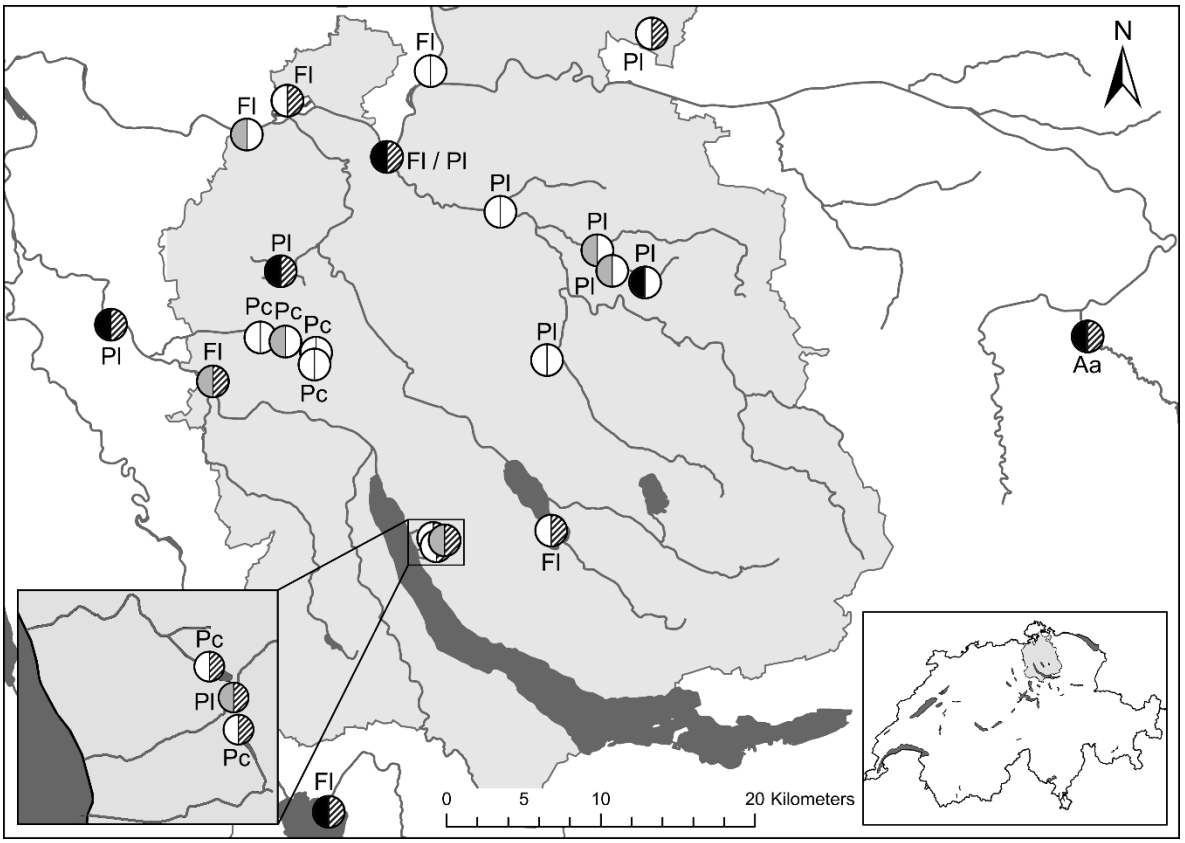


Figure 2. Map of sampling sites and *A. astaci* occurrence (dashed grey = present in crayfish tissue, black = present in eDNA, light grey = present in eDNA below LOD. Labels indicate initials of the crayfish species present at each location (Aa = *A. astacus*, Fl = *F. limosus*, Pc = *P. clarkii*, Pl = *P. leniusculus*). The grey area of the map indicates borders of Canton Zurich.

Hierarchical occupancy models with constant parameters estimated occupancy probability of *A. astaci* in water per site to be $\Psi(.) = 0.314$ in invasive crayfish population sites. The estimate of parasite detection per sample was $\theta(.) = 0.366$ and $p(.) = 0.726$ per qPCR replicate. Thus, to reach detection rates per site of 95 % or above, seven water samples would need to be taken. Model fit did not improve much when additional variables were included. If detections of *A. astaci* concentrations below LOD were considered, the occupancy

probabilities increased to $\Psi(.) = 0.544$, $\theta(.) = 0.760$ and $p(.) = 0.940$ and only three water samples would need to be taken for detection success to exceed 95 %. All tested models are listed in Table S2 in the Supplement.

Detection of *A. astaci* in water samples was more likely with increasing parasite prevalence in the crayfish population ($\chi^2 = 4.042$, df = 1, p = 0.044). Furthermore, there was a positive effect of the total number of infected crayfish on eDNA detection success ($\chi^2 = 4.497$, df = 1, p = 0.034). Parasite detection rates in water were neither affected by highest parasite agent levels in the crayfish per population ($\chi^2 = 5.417$, df = 3, p = 0.144) nor by invasive crayfish species ($\chi^2 = 3.663$, df = 2, p = 0.160). Further, we did not observe associations of mean *A. astaci* concentration in water with parasite prevalence in invasive crayfish ($F_{1,9} = 1.521$, p = 0.249), nor with maximum parasite agent levels ($F_{3,8} = 0.753$, p = 0.551) or mean *A. astaci* concentration estimates in crayfish tissues (Fig. 3; abdominal cuticle: $F_{1,6} = 0.985$, p = 0.359; uropod: $F_{1,6} = 1.376$, p =0.285).

A. astaci detection and concentrations in different types of crayfish tissue

Among the *A. astaci* positive invasive crayfish (n = 86), the parasite DNA was detected in both tissue types in 44.2 % (n = 38) of the crayfish, and in the remaining crayfish, 24 were *A. astaci* positive in abdominal cuticle tissue and 24 in uropod tissue. The detection success in either tissue type of invasive crayfish was not affected by species (abdominal cuticle: $\chi^2 = 1.331$, df = 2, p = 0.214; uropod: $\chi^2 = 1.678$, df =2, p = 0.432), nor sex (abdominal cuticle: $\chi^2 = 0.226$, df = 1, p = 0.635; uropod: $\chi^2 = 0.002$, df = 1, p = 0.961), nor size (abdominal cuticle: $\chi^2 = 0.021$, df = 1, p = 0.886; uropod: $\chi^2 = 0.065$, df = 1, p = 0.799) nor any interaction effects. There was a marginally significant interaction effect between sex and size for detection probability in abdominal cuticle samples only ($\chi^2 = 3.917$, p = 0.048), which indicated that detection rates were higher for larger females and smaller males.

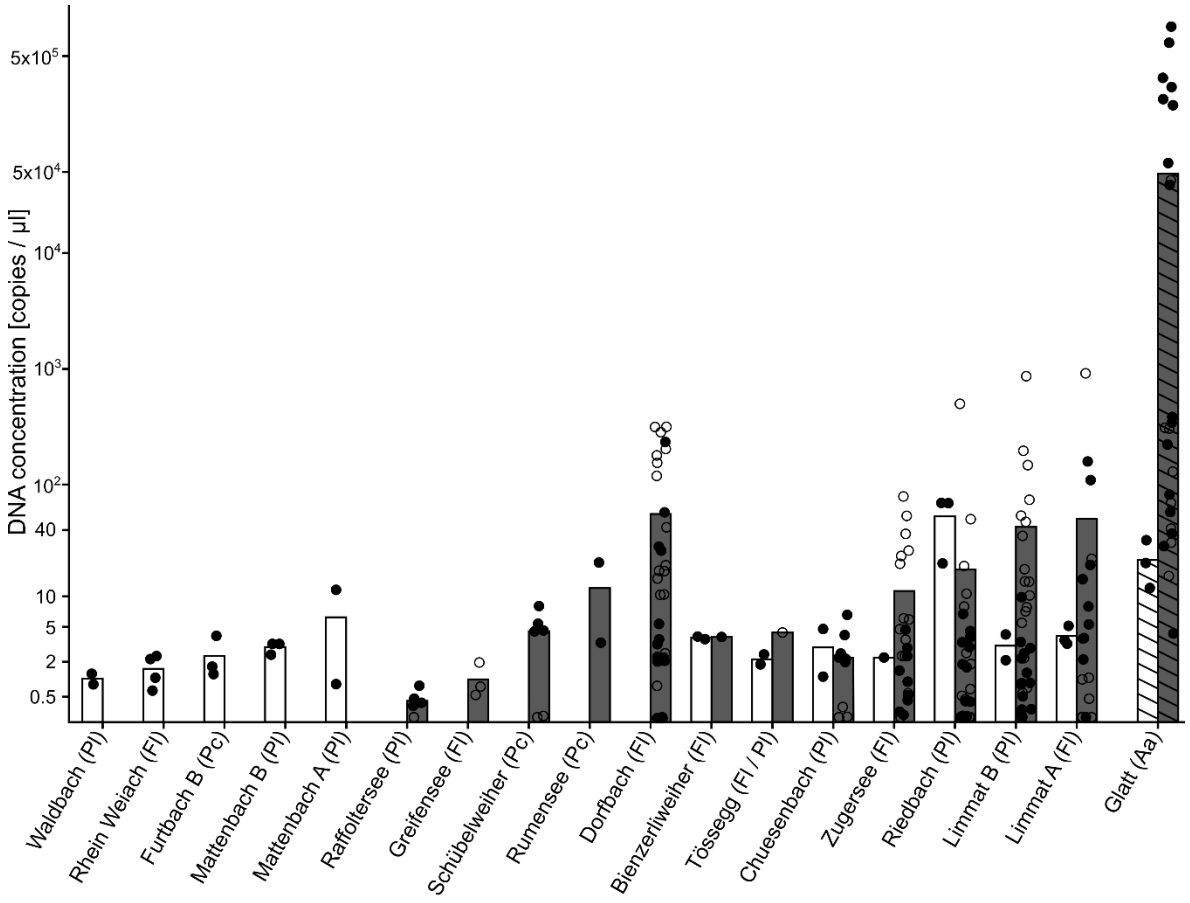


Figure 3. Mean *A. astaci* concentrations in crayfish tissue (grey bars) and / or water (white bars) per sampling site. The bars are labelled with site names (see Table 1 for more details), followed by abbreviations indicating the crayfish species in brackets (Aa = *A. astacus*, Fl = *F. limosus*, Pc = *P. clarkii*, Pl = P. *leniusculus*). Dots show *A. astaci* concentrations per crayfish and water sample (in crayfish samples black dot = abdominal cuticle, empty = uropod). The active *A. astaci* outbreak site "Glatt" is distinguishable by the hashed bar pattern.

Estimated *A. astaci* concentrations differed significantly between tissue types of the same crayfish, with concentrations of uropods being higher on average ($Z = -2.927$, $p = 0.003$), but these concentrations did not correlate significantly ($R = 0.126$, $F_{1,53} = 0.86$, $p = 0.358$), even when we excluded 42 individuals for which the two types of tissue samples were not extracted with the same method ($R = 0.240$, $F_{1,44} = 2.695$, $p = 0.108$). Estimated *A. astaci* DNA concentrations in abdominal cuticle samples of invasive crayfish differed among species ($\chi^2 = 7.656$, $df = 2$, $p = 0.022$), with *P. clarkii* showing the highest and *P. leniusculus* the lowest concentrations on average. The other main effects were not significant (size: $\chi^2 = 0.948$, $df = 1$, $p = 0.330$; sex: $\chi^2 = 0.989$, $df = 1$, $p = 0.320$), but there was a significant interaction between species and size ($\chi^2 = 7.942$, $df = 2$, $p = 0.019$) with *F. limosus* showing decreasing parasite DNA concentrations with increasing size, while *P. leniusculus* and

P. clarkii demonstrated parasite DNA concentrations slightly increasing with size. Furthermore, a significant interaction between sex and size (χ^2 = 5.411, df = 1, p = 0.020) was noted, with parasite DNA concentrations in females slightly increasing, and concentrations in males slightly decreasing with size. Concentrations of *A. astaci* DNA in uropod tissues of invasive crayfish were not affected by species (χ^2 = 2.852, df = 2, p = 0.240), sex (χ^2 = 0.072, df = 1, p = 0. 789), or size (χ^2 = 0.060, df = 1, p = 0.806) and the variables did not show significant interaction effects.

Native noble crayfish from the single outbreak site showed strong *A. astaci* signals above LOD and also water samples from this site were clearly positive for the crayfish plague. The diseased *A. astacus* also showed the highest semi-quantitative *A. astaci* agent levels we found in this study (A5), while the highest levels in the invasive species were A3 for *F. limosus* and *P. leniusculus* and A2 for *P. clarkii* (Tab. 1).

PCR inhibition in tissue and eDNA samples

None of the eDNA samples were strongly inhibited (all IPC Δ Cq < 1) and *A. astaci* detection success in water samples was not affected by PCR inhibition (χ^2 = 1.02, p = 0.313). On the other hand, part of the DNA extractions from crayfish tissue samples (8.3 % of cuticle samples, 24.9 % of uropod samples) showed substantial signs of inhibition, i.e. IPC Δ Cq > 3, and 9.9 % of uropod tissue samples failed to amplify the IPC at all (Fig. 4). Inhibition estimated as Δ Cq correlated between abdominal cuticle and uropod tissues of the same individuals ($F_{1,389}$ = 61.410, p < 0.001) and reached higher levels in uropod samples (Z = - 2.589, p = 0.01). Furthermore, inhibition in tissue samples of invasive crayfish increased with crayfish size (abdominal cuticle: R = 0.197, $F_{1,426}$ = 17.213, p < 0.001; uropod: R = 0.369, $F_{1,379}$ = 42.665, p < 0.001) and differed between species, being highest in *P. leniusculus* (abdominal cuticle: Kruskal-Wallis χ^2 = 106.16, df = 2, p < 0.001; uropod: Kruskal-Wallis χ^2 = 103.11, df = 2, p < 0.001).

Even though the IPC indicated PCR inhibition for part of the DNA extractions obtained from crayfish tissue samples, this inhibition did not affect *A. astaci* detection success significantly (abdominal cuticle: χ^2 = 0.724, df = 1, p = 0.395; uropod: χ^2 = 0.313, df = 1, p = 0.576). Accordingly, *A. astaci* DNA concentrations in tissue samples of invasive crayfish were not affected by inhibition either, neither for abdominal cuticle ($F_{1,79}$ = 0.524, p = 0.471) nor uropod tissue ($F_{1,80}$ = 2.971, p = 0.089).

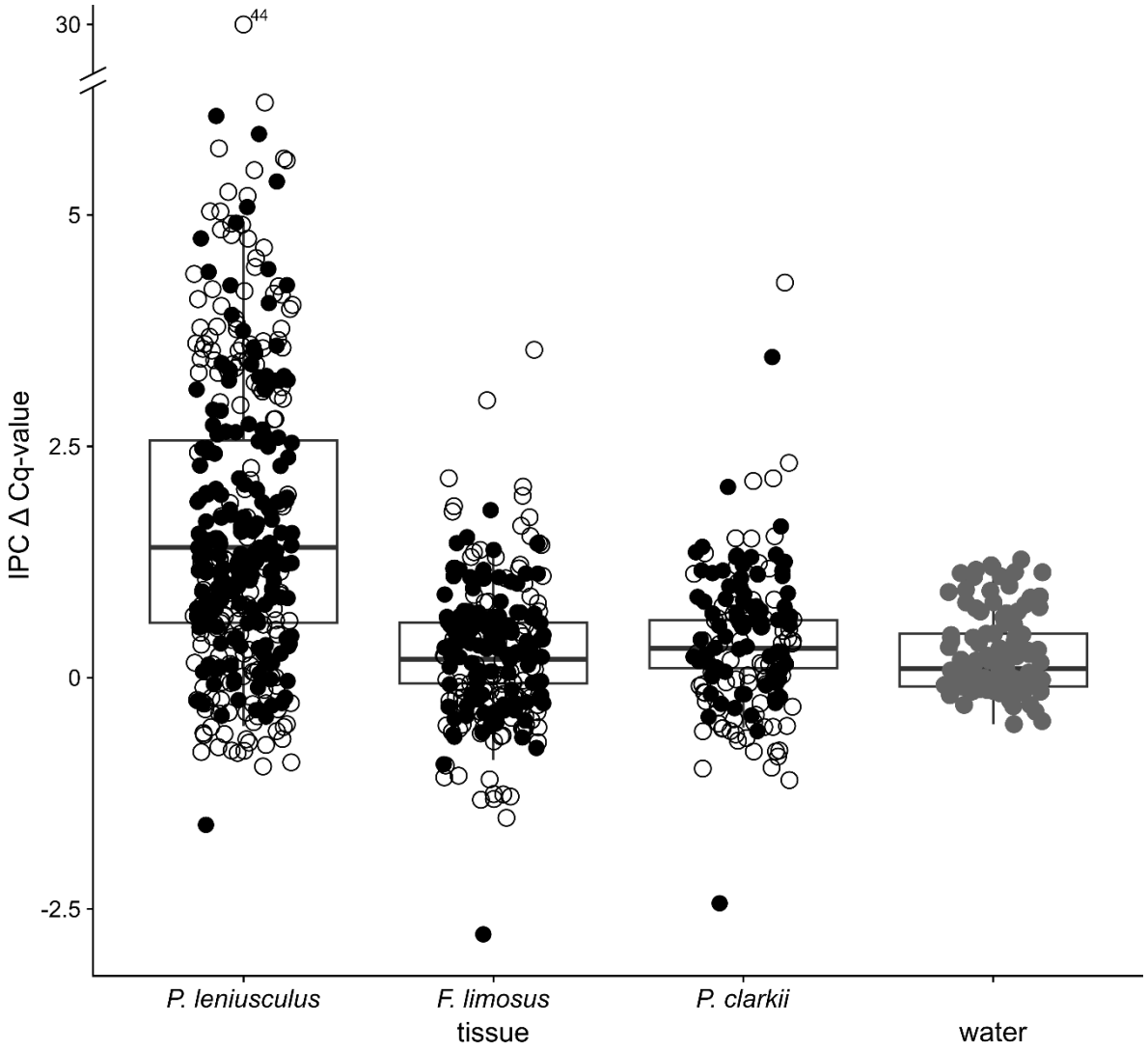


Figure 4. Inhibition score Δ Cq for three invasive crayfish species and water samples. Black dots=abdominal cuticle, empty dots=uropod tissue samples. A total of 44 uropod tissue samples failed to amplify the IPC and are visualised as a single dot at IPC Δ Cq-values = 30.

Discussion

Aphanomyces astaci detection success in water and crayfish tissue

We investigated the occurrence of crayfish plague agent *Aphanomyces astaci* in invasive crayfish populations in an area in Northern Switzerland, using molecular detection methods in crayfish tissue and ambient water samples. Considering both methods, 13 of 23 sampled invasive crayfish populations clearly harboured *A. astaci*, confirming the disease agent reservoir status in around half of the sampled sites. *A. astaci* was detected in crayfish tissue in twelve and in water in five of the 23 surveyed invasive crayfish populations (Table 1; Fig. 2), making the tissue sampling method more successful in detecting the parasite than the

eDNA method. If weak signals below LOD are considered as true positives, the number of sites with *A. astaci* detections in water or tissue increases to 17, with detection in water in a total of twelve sites, but even then the parasite was detected by both methods in seven sites only. We argue that while we implemented an LOD in this study, the detections below LOD should not be disregarded categorically, since they could likely indicate low levels of parasite DNA in water. As eDNA samples often contain low starting amounts of target DNA, the frequently applied 95 % detection threshold, stemming for guidelines for mainly gene expression assays (Bustin et al. 2009), have been challenged for its suitability for eDNA (Hunter et al. 2017, Klymus et al. 2019). The LOD in this study was defined at a level where 62.5% of replicates amplified and we stress that interpretation of results and LOD may be specific to assay conditions. For example, in this study a modification (Strand et al. 2011, 2014) of the original assay from Vrålstad et al. (2009) was used to improve specificity by increasing the annealing temperature, with a trade-off with sensitivity. Therefore, when assay specificity is very high, evaluating detections below LOD is important, particularly when applied to a deadly pathogen like the crayfish plague.

The infection intensity in most invasive crayfish in Europe is low, making it challenging to confirm disease agent-free status of a population. The highest agent level observed in tissue of an invasive crayfish in this study was A3 which is comparable to levels found by Vrålstad et al. (2009) but lower than in Vrålstad et al. (2011), Filipová et al. (2013) and Wittwer et al. (2018). Therefore, we suggest that variable detection may be a characteristic of asymptomatic carrier populations, and employment of multiple methods will be required to ensure disease-free status of crayfish populations. In the following sections we further discuss how the variation in detection could arise due to several factors.

Factors influencing *A. astaci* detection in crayfish tissues

DNA of *A. astaci* was not detected in crayfish tissue in 5 sites where DNA was found in water samples, although only one of these eDNA results was above LOD. These results indicate that the tissue sampling method failed to detect *A. astaci* in some infected populations, unless the eDNA at these sites originated from unsampled infected populations upstream. Possibly, our sample sizes were insufficient for sites with low prevalence of the parasite. Calculations by Schrimpf et al. (2013) showed that, depending on test sensitivity (detection success rate per individual) and population size, 34 to almost a 1000 crayfish need

to be sampled for reliable detection (≥ 95 % success rate) in populations with low *A. astaci* prevalence, i.e. 10 % or less infected individuals.

Detection rates in samples from the abdominal cuticle and uropods were equally high in our study, but the overlap was only partial. In 48 of the 86 invasive crayfish positive for *A. astaci*, the parasite DNA was detected in only one of the two tissue types. Sampling two different tissue types thus more than doubled the detection rate, similar to observations by Oidtmann et al. (2006). Detection rates might be improved further if additional parts of the crayfish cuticle are analysed, e.g. the whole soft abdominal cuticle or walking legs (Vrålstad et al. 2011). Individual level variation in site of infection is to be expected as *A. astaci* infections mostly occur in spots where the epicuticle, the outermost layer of the exoskeleton, is absent or damaged (Unestam & Weiss 1970). Crayfish that tested positive for both tissue types in this study generally contained higher *A. astaci* DNA concentrations in the uropods than the abdominal cuticle. Vrålstad et al. (2011) discussed the higher degree of exposure to zoospores and larger total exposure area of the uropods compared to the abdominal cuticle as likely reasons for the observed higher parasite concentrations.

Although infection intensity is potentially influenced by host life-history variation, we found little evidence of it affecting detection of *A. astaci* in analysed crayfish. For example, there was no general increase in detection rate with crayfish size, even though larger individuals yielded larger uropod tissue samples. Sex and species did not influence parasite detection rate, either. However, a significant interaction term indicated that detection rates increased slightly with size in females but not in males. In contrast, Vrålstad et al. (2011) observed higher *A. astaci* detection rates in females and large crayfish of both sexes. However, comparisons of studies are difficult as we analysed individuals belonging to different species and originating from multiple populations, while Vrålstad et al. (2011) analysed these patterns in a single large lake population of *P. leniusculus*, where crayfish are all exposed to similar environmental conditions and infection risks. We did indeed observe significant differences among invasive crayfish species in *A. astaci* concentrations estimated from abdominal cuticle samples. There were also significant interactions between species and size as well as sex and size, which we find difficult to explain biologically. Given that spore release and infection intensity may be influenced by the molting cycle (Svoboda et al. 2013), it may have mattered that we conducted sampling at only one time point, potentially biasing results through moult cycle differences. Juvenile crayfish moult often, adult males and

females normally moult once, or occasionally twice a year, females usually after releasing their young in late summer (Westman & Savolainen 2002).

Crayfish plague detection may also be hampered by PCR inhibition. While eDNA samples did not indicate any relevant levels of inhibition, inhibition was observed in some extractions from crayfish tissues (IPC Δ Cq > 3), especially from uropods. However, the magnitude of IPC inhibition was not associated with parasite detection success. These results suggest that although PCR inhibition may occur in some samples, the qPCR assay employed for *A. astaci* is robust and not significantly influenced by such effects. Nevertheless, it cannot be excluded that inhibition could have influenced *A. astaci* concentration estimates from crayfish samples, further emphasizing the importance of sampling multiple tissue samples and individuals.

Reasons for variation in eDNA detection

A. astaci was clearly detected in ambient water in only four of twelve infected invasive crayfish populations (in seven populations if signals below LOD are considered). This contrasts with previous studies which report up to 100% success of eDNA sampling in infected crayfish populations (Strand et al. 2014, Wittwer et al. 2018, 2019). The difference could be related to pathogen prevalence and/or sampling effort. Wittwer et al. (2018, 2019) investigated streams harbouring invasive crayfish populations with infection prevalences of ≥ 60 % and took up to 32 eDNA samples per stream, while Strand et al. (2014) collected ten 15 L water samples from lakes containing *P. leniusculus* with ≥ 50 % infection prevalence. *A. astaci* prevalence in in this study was as low as 4 % (Rhein Tössegg), with less than half the populations showing prevalences above 50 % (Table 1), and we collected three 5 L water samples per site. Indeed, our *A. astaci* detection success in water increased with higher *A. astaci* prevalence and the absolute number of infected crayfish and it was detected in water from three of the four sites harbouring crayfish populations with > 50% infection prevalence. Clearly, the amount of pathogen spores in the water not only depends on prevalence but also on host population density, which we were not able to quantify accurately due to the different types of waterbodies surveyed (small streams, large rivers, lakes and ponds) and the different methods of crayfish collection. This may also explain the lack of a quantitative association between estimated *A. astaci* DNA concentrations in water and estimated concentrations / agent levels in crayfish tissues. Overall, these results imply that an increased eDNA sampling effort may improve reliability of *A. astaci* detection in invasive crayfish populations with a

low infection prevalence. According to the occupancy modeling results, seven water samples need to be taken per site for a 95 % and higher chance of detecting *A. astaci* concentrations above the LOD in water. However, only three water samples are needed if concentrations below the LOD are considered sufficient for positive detection. Since sampling effort should be reasonable to keep large-scale surveys achievable and cost-effective, it is worth also considering other aspects that could improve reliability. The timing of a survey is a crucial factor for *A. astaci* detection success in water. In this study, eDNA samples were collected May to September. *A. astaci* concentrations in water have shown increased levels during crayfish moulting stages in aquaria experiments (Svoboda et al. 2013). Adult crayfish usually moult only once or twice a year, mostly when water temperatures are high during summer (Westin & Gydemo 1986), which indicates our time of sampling was appropriate for increasing *A. astaci* detection rates. However, Wittwer et al. (2018) took monthly eDNA samples from several sites throughout a year and measured highest *A. astaci* concentrations in October, coinciding with the mating season when crayfish show increased aggressive behaviour towards each other, which frequently leads to injuries. Due to geographical vicinity of the study system (Germany), we can expect similar seasonal dynamics of *A. astaci* concentrations in water, which indicate *A. astaci* eDNA surveys should be conducted later in the year than in this study, i.e. September to October, to maximise detection success and therefore, reliability of the results.

Outbreak site results

All tissue samples of the eight *A. astacus* individuals and the water samples collected at an active crayfish plague outbreak site (Fig. 2) were found positive for *A. astaci*. The tissue samples had highest parasite concentrations and, therefore, agent levels (A5), of all the collected crayfish in this study and parasite eDNA concentrations were second highest of all surveyed sites (highest in Riedbach; Table 1; Fig. 3). The same river was sampled downstream from the outbreak site for another survey a month before the outbreak was noticed, and *A. astaci* eDNA was already found at around a third of the concentrations measured during the outbreak (pers. obs. N. Sieber). While this was one site only, these results suggest that the eDNA method works reliably when parasite loads in a population and therefore in water, are high. The challenges arise from the low amount of parasite spores released by the highly resistant invasive crayfish populations (Strand et al. 2014).

Conclusion

This survey of the crayfish plague agent in asymptomatic invasive crayfish populations shows that two different monitoring methods convey a different picture of *A. astaci* occurrence. In many cases when crayfish plague infection intensities and prevalence are low, concluding the absence of the plague from a negative result of either method would be misleading. Avenues for optimization of both detection methods are identified. For eDNA-based detection, higher sampling effort would increase detection reliability of asymptomatic crayfish populations. For detection of the parasite in crayfish tissue, analysis of larger numbers of crayfish and more parts of the crayfish cuticle, e.g. the whole abdominal cuticle or leg joints. Both methods would benefit from aligning the time of sampling to seasonal dynamics of the parasite, determined by both host and parasite ecology. Repeated sampling of the same sites during the appropriate season could further improve reliability of the detection result. Decisions on monitoring methods not only depend on reliability of the method, but also on cost and effort, and the ultimate aim of the monitoring and surveillance activity. The effort and cost required for the crayfish tissue sampling method and its suggested improvements is substantially higher than for the eDNA water sampling method. Regular monitoring with the crayfish tissue sampling method alone might therefore not be feasible. Thus, a combination of the two methods would facilitate more frequent monitoring campaigns and deliver more accurate knowledge of occurrence and spread of the crayfish plague for the implementation of effective management strategies.

Acknowledgements We thank Raffael Stegmayer and Pravin Ganesanandamoorthy for their help extracting crayfish. We thank Pascal Reichlin and Alexandre Gouskov for providing crayfish for the study and Andreas Hertig of the cantonal fishing and hunting administrations (Fischerei- und Jagdverwaltung) for the permit for capturing crayfish. We further thank Bettina Dubach, Pravin Ganesanandamoorthy, Marta Reyes, Dr. Robert Dünner and Jana Jucker for their assistance in the field. Finally, we thank Prof. Dr. Jukka Jokela who funded a significant part of the last year of my PhD.

Funding This study was funded by Eawag, the Swiss Federal Institute of Technology Zürich (ETH Zürich) and the Swiss Federal Office for the Environment (FOEN).

Conflicts of interest The authors declare that they have no conflict of interest.

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data availability Datasets generated and/or analysed for this study are available on dryad: https://datadryad.org/stash/share/hxp76PQxR7k_dlw6ifW4CRRu8K3Nwfzc_k0sCMLMjWk

Author's contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Natalie Sieber, Hanna Hartikainen and Christoph Vorburger. The first draft of the manuscript was written by Natalie Sieber and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Literature cited

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. OIE Rev Sci Tech 15:603–632

Alderman DJ, Polglase JL, Frayling M (1987) *Aphanomyces astaci* pathogenicity under laboratory and field conditions. J Fish Dis 10:385–393

Bass D, Stentiford GD, Littlewood DTJ, Hartikainen H (2015) Diverse applications of environmental DNA methods in parasitology. Trends Parasitol 31:499–513

Blodgett R (2010) Bacteriological Analytical Manual (BAM) Appendix 2: Most probable number from serial dilutions. Food and Drug Administration. https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions (accessed 24 July 2020)

Bohman P, Nordwall F, Edsman L (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. Bull Fr la Pêche la Piscic 380–381:1291–1302

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Carraro L, Bertuzzo E, Mari L, Fontes I, Hartikainen H, Strepparava N, Schmidt-Posthaus H, Wahli T, Jokela J, Gatto M, Rinaldo A (2017) Integrated field, laboratory, and theoretical study of PKD spread in a Swiss prealpine river. Proc Natl Acad Sci USA 114:11992–11997

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. Cell Microbiol 5:353–357

Dorazio RM, Erickson RA (2018) Ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380

Dunn AM, Hatcher MJ (2015) Parasites and biological invasions: parallels, interactions, and control. Trends Parasitol 31:189–199

Elmiger C, Gouskov A, Philipp U, Hertig A (2018) Flusskrebs-Managementplan Kanton Zürich. Pachtperiode 2018-2026. Zürich.

Filipová L, Petrusek A, Matasová K, Delaunay C, Grandjean F (2013) Prevalence of the Crayfish Plague Pathogen *Aphanomyces astaci* in Populations of the Signal Crayfish *Pacifastacus leniusculus* in France: Evaluating the Threat to Native Crayfish. PLoS One 8:e70157

Gelfand AE, Ghosh SK (1998) Model choice: a minimum posterior predictive loss approach. Biometrika 85:1–11

Ghanawi J, Saoud G, Zakher C, Monzer S, Saoud IP (2019) Clove oil as an anaesthetic for Australian redclaw crayfish *Cherax quadricarinatus*. Aquac Res 50:3628–3632

Hochwimmer G, Tober R, Bibars-Reiter R, Licek E, Steinborn R (2009) Identification of two GH18 chitinase family genes and their use as targets for detection of the crayfish-plague oomycete *Aphanomyces astaci*. BMC Microbiol 9:1–17

Holdich DM (2003) Crayfish in Europe - an overview of taxonomy, legislation, distribution, and crayfish plague outbreaks. Proceedings of a conference held on 7th November, 2002. In: *Management & Conservation of Crayfish*. Holdich DM, Sibley PJ (eds) Bristol, p 15–34

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl Manag Aquat Ecosyst 2009:394–395

Hunter ME, Dorazio RM, Butterfield JSS, Meigs-Friend G, Nico LG, Ferrante JA (2017) Detection limits of quantitative and digital PCR assays and their influence in presence – absence surveys of environmental DNA. Mol Ecol Resour 17:221–229

Klymus KE, Merkes CM, Allison MJ, Jackson CA, Goldberg CS, Helbing CC, Hunter ME, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter CA (2019) Reporting the limits of detection and quantification for environmental DNA assays. Environ DNA 00:1–12

Kokko H, Koistinen L, Harlioğlu MM, Makkonen J, Aydin H, Jussila J (2012) Recovering Turkish narrow clawed crayfish (*Astacus leptodactylus*) populations carry

Aphanomyces astaci. Knowl Manag Aquat Ecosyst 404:12p1-12p7

Kouba A, Petrusek A, Kozák P (2014) Continental-wide distribution of crayfish species in Europe: Update and maps. Knowl Manag Aquat Ecosyst 413:05p1-05p31

Kozubíková E, Filipová L, Kozák P, Ďuriŝ Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B, Petrusek A (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech republic. Conserv Biol 23:1204–1213

Lowe S, Browne M, Boudjelas S, De Poorter M (2000) 100 of the world's worst invasive alien species. A selection from the global invasive species database. The Invasive Species Specialist Group (ISSG), Auckland, New Zealand.

Nyhlén L, Unestam T (1980) Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. J Invertebr Pathol 36:187–197

Oidtmann B, Bausewein S, Hölzle L, Hoffmann R, Wittenbrink M (2002) Identification of the crayfish plague fungus *Aphanomyces astaci* by polymerase chain reaction and restriction enzyme analysis. Vet Microbiol 85:183–194

Oidtmann B, Geiger S, Steinbauer P, Culas A, Hoffmann RW (2006) Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. Dis Aquat Org 72:53–64

Oidtmann B, Schaefers N, Cerenius L, Söderhäll K, Hoffmann RW (2004) Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. Vet Microbiol 100:269–282

Price PW, Westoby M, Rice B (1988) Parasite-mediated competition: some predictions and tests. 131:544–555

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.r-project.org/

Reynolds JD (1988) Crayfish extinctions and crayfish plague in central Ireland. Biol Conserv 45:279–285

Schrimpf A, Maiwald T, Vrålstad T, Schulz HK, Śmietana P, Schulz R (2013) Absence of the crayfish plague pathogen (*Aphanomyces astaci*) facilitates coexistence of

European and American crayfish in central Europe. Freshw Biol 58:1116–1125

Schrimpf A, Pârvulescu L, Copilaş-Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta - A potential threat to freshwater biodiversity in southeastern Europe. Aquat Invasions 7:503–510

Sieber N, Hartikainen H, Vorburger C (in press) Validation of an eDNA-based method for the detection of wildlife pathogens in water. Dis Aquat Org

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Dis Aquat Org 95:9–17

Strand DA, Jussila J, Johnsen SI, Viljamaa-Dirks S, Edsman L, Wiik-Nielsen J, Viljugrein H, Engdahl F, Vrålstad T (2014) Detection of crayfish plague spores in large freshwater systems. J Appl Ecol 51:544–553

Strand DA, Jussila J, Viljamaa-Dirks S, Kokko H, Makkonen J, Holst-Jensen A, Viljugrein H, Vrålstad T (2012) Monitoring the spore dynamics of *Aphanomyces astaci* in the ambient water of latent carrier crayfish. Vet Microbiol 160:99–107

Strauss A, White A, Boots M (2012) Invading with biological weapons: the importance of disease-mediated invasions. Funct Ecol 26:1249–1261

Strayer DL (2010) Alien species in fresh waters: Ecological effects, interactions with other stressors, and prospects for the future. Freshw Biol 55:152–174

Stucki P, Zaugg B (2011) Aktionsplan Flusskrebse Schweiz. Artenförderung von Edelkrebs, Dohlenkrebs und Steinkrebs. Bern.

Stucki P, Zaugg B (2005) Decapoda. Fauna Helvetica 15. Centre Suisse de Cartographie de la Faune, Neuchâtel.

Svoboda J, Kozubíková-Balcarová E, Kouba A, Buřič M, Kozák P, Diéguez-Uribeondo J, Petrusek A (2013) Temporal dynamics of spore release of the crayfish plague pathogen from its natural host, American spiny-cheek crayfish (*Orconectes limosus*), evaluated by transmission experiments. Parasitology 140:792–801

Thomsen PF, Willerslev E (2015) Environmental DNA - an emerging tool in conservation

for monitoring past and present biodiversity. Biol Conserv 183:4–18

Unestam T, Weiss DW (1970) The host-parasite relationship between freshwater crayfish and the crayfish disease fungus *Aphanomyces astaci*: responses to infection by a susceptible and a resistant species. J Gen Microbiol 60:77–90

Vrålstad T, Johnsen SI, Fristad RF, Edsman L, Strand D (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. Dis Aquat Org 97:75–83

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:146–155

Watanabe S (2010) Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. J Mach Learn Res 11:3571–3594

Westin L, Gydemo R (1986) Influence of light and temperature on reproduction and moulting frequency of the crayfish, Astacus astacus l. Aquac 52:43–50

Westman K, Savolainen R (2002) Growth of the signal crayfish, *Pacifastacus leniusculus*, in a small forest lake in Finland. Boreal Environ Res 7:53–61

Wittwer C, Stoll S, Strand D, Vrålstad T, Nowak C, Thines M (2018) EDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia 807:87–97

Wittwer C, Stoll S, Thines M, Nowak C (2019) EDNA-based crayfish plague detection as practical tool for biomonitoring and risk assessment of *A. astaci*-positive crayfish populations. Biol Invasions 21:1075–1088

Supplementary Material Chapter 2

Text S1. Crayfish extraction DNeasy Blood and Tissue Kit

1. Put tissue samples in 2ml tubes with 1 large steel bead.
2. Freeze tissue samples (-80°C, minimum 10 minutes).
3. Bead beat samples at full speed for 30 seconds (if not properly crushed, repeat).
4. Thaw tissue samples on 56°C.
5. Add 800µl ATL buffer to the samples.
6. Bead beat at full speed for 30 seconds.
7. Spin for 1 minute to remove foam.
8. Freeze (-80°C, minimum 10 minutes) and thaw samples at 56°C, spin down.
9. Add 10µl proteinase K (20 mg/ml), vortex and incubate at 56°C overnight (note time).
10. Centrifuge 5 minutes at 12’000 x g.
11. Transfer 550µl supernatant to a new 2ml tube.
12. Add 550µl Buffer AL. Mix thoroughly by vortexing.
13. Incubate samples at 56°C for 10 minutes.
14. Add 550µl ethanol (96-100%). Mix thoroughly by vortexing.
15. Transfer 650µl to a DNeasy Mini spin column placed in a 2ml collection tube. Centrifuge at ≥ 6000 x g for 1 minute. Discard the flow-through. Repeat until all liquid has been processed.
16. Discard the collection tube and place the spin column in a new 2ml collection tube.
17. Add 500µl Buffer AW1. Centrifuge at ≥ 6000 x g for 1 minute. Discard the flow-through and collection tube. Place the spin column in a new 2ml collection tube.
18. Add 500µl Buffer AW2. Centrifuge at 20’000 x g for 3 minutes. Discard the flow-through and collection tube.
19. Transfer the spin column to a new 1.5ml tube.
20. Elute the DNA by adding 200µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature. Centrifuge at ≥ 6000 x g for 1 minute.

Text S2. Crayfish tissue high salt extraction

1. Put all tissue sample on ice.
2. Add one steel bead in a 2ml tube.
3. Take the crayfish tissue sample and put it into the 2ml tube.
4. Put the tubes for 30 seconds in liquid nitrogen.
5. Bead beat samples at full speed for 30 seconds (if not properly crushed, repeat).
6. Add 800µl TNES buffer and 20µl proteinase K (10 mg/ml).
7. Incubate tubes for 3h at 65°C and 1000 x rpm.
8. Add 230 µl 5M NaCl and shake hard for 15 seconds.
9. Put tubes for 5min on ice (optional but recommended).
10. Centrifuge tubes at full speed for 10 min at room temperature.
11. Transfer supernatant in a new 2ml tube.
12. Add 150 µl 5M NaCl and shake hard for 15 seconds.
13. Put tubes on ice for 5 min (optional but recommended).
14. Centrifuge tubes at full speed for 10 min at room temperature.
15. During centrifugation time add 1ml ice cold 100% EtOH in a 2ml tube
16. Transfer supernatant to the 2ml tube with EtOH.
17. Invert few times and centrifuge for 10-15 min at full speed and 4°C.
18. Discard supernatant and add 500 µl ice cold 70% EtOH.
19. Invert few times and centrifuge for 10-15 min at full speed and 4°C.
20. Discard supernatant and let pellet dry.
21. Resuspend pellet with 150 µl TE-buffer.

Text S3. Crayfish tissue extraction with CTAB

1. Put tissue samples into sturdy 2 ml tubes with 3-4 steel beads.
2. Freeze samples at -80°C for a minimum of 15 min.
3. Add 700 µl pre-heated (65°C) CTAB buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na2EDTA).
4. Homogenise samples: 3x 6500 rpm for 1 min with 2 min rest time in between.
5. Freeze samples at -80°C for a minimum of 10 min.
6. Incubate at 65°C for 5-10 min.
7. Centrifuge for 5 min to remove foam.
8. Add 10 µl RNase solution (10 mg/ml).
9. Mix and incubate at 65°C for 30 min.
10. Add 10 µl proteinase K (20 mg/ml).
11. Mix and incubate at 65°C for 30 min.
12. Vortex and centrifuge at 12’000 x g for 5 min.
13. Transfer 600 µl of supernatant to new 1.5 ml tubes.
14. Add 600 µl of chloroform and mix by vortexing.
15. Centrifuge at 18’000 x g for 15 min.
16. Carefully transfer 400 µl of supernatant to new 1.5 ml tubes.
17. Add 300 µl of ice-cold isopropanol and mix by careful inversion.
18. Incubate at room temperature for 20 min.
19. Centrifuge at 18’000 x g for 10 min to pellet DNA.
20. Discard supernatant without disturbing the pellet.
21. Add 300 µl ice-cold 70% EtOH.
22. Centrifuge at 18’000 x g for 5 min.
23. Discard supernatant without disturbing the pellet.
24. Dry pellet for 15-30 min (heat block 50°C or vacuum dryer).
25. Re-suspend in TE-buffer.
26. Leave at room temperature for minimum 1h to dissolve pellet, then freeze at -20°C.

5’-
AAGGCTTGTGCTGGGATGTTC***TTCGGGACGACCC***GGC**TAGCAGAAGGTTTCGCAA GAAG**TTTTTCCTTGATATAATACAGTGTGCCATATGTCACGAGTCGAACAAAATTTATT TATTTTTTCGACAAATTAATTGGAAATTGAATAATTTAATTGAAAAAAATTGAAAATAAA TATTAAAAACAACTTTTGACAACGGATCTCTTGGCTTTTTTAGAGCAAATCGCGGTAGT TTTGCTTGTACTTCGGTACGAGTGGACACATATTGCTTTTTGTGATTTCTGCGAGTCT GTTGTCAAAGTACAAGGCACGTAAGGAGAGTTGGTATGCTGGTGCATTTCTTTTTTGG TTGTTTAGTTTGGGCTCACCATATGTATGTTGTTGGTTTAGACACTGATACAAGATCTT ATTTTATGGCTGCCACTATGACAATAGCTGTCCCTACAGGGA -3’

Figure S1. Sequence of double-stranded Gblocks fragment used to create the standard curve for quantitative PCR. Forward and reverse primers are in bold, probes in bold and italic for *A. astaci*.

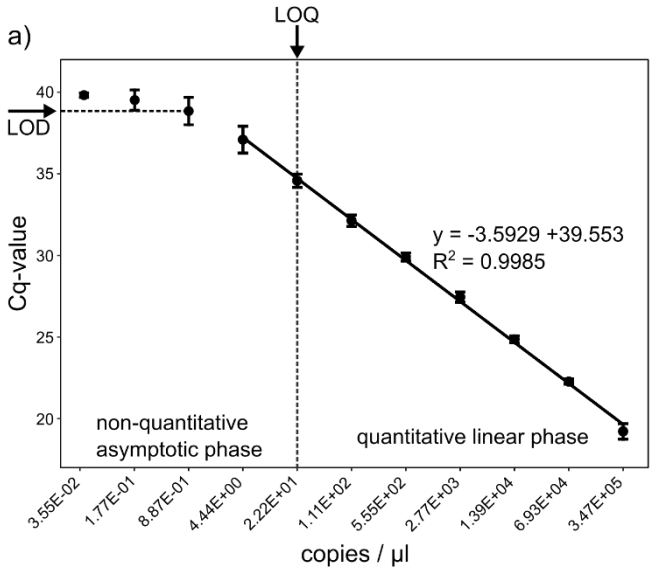


Figure S2. *A. astaci* standard curve used for defining the limit of detection (LOD) and limit of quantification (LOQ).

Table S1 Dilution series of Gblocks fragment for absolute quantification and determination of limit of detection (LOD). Standards 5 to 11 were run in 30, and standards 12 to 15 in 40 replicates. Mean Cq-values and detection rates are calculated from all replicates.

Standard	Dilution	copies µl^{-1}	nr. of replicates	mean Cq-values ± SD	% detection
5	5^-5	3.47E+05	30	19.22 ± 0.48	100
6	5^-6	6.93E+04	30	22.28 ± 0.15	100
7	5^-7	1.39E+04	30	24.85 ± 0.21	100
8	5^-8	2.77E+03	30	27.45 ± 0.31	100
9	5^-9	5.55E+02	30	29.91 ± 0.25	100
10	5^-10	1.11E+02	30	32.13 ± 0.35	100
11	5^-11	2.22E+01	30	34.58 ± 0.41	100
12	5^-12	4.44E+00	40	37.09 ± 0.83	100
13	5^-13	8.87E-01	40	38.84 ± 0.84	62.5
14	5^-14	1.77E-01	40	39.52 ± 0.62	22.5
15	5^-15	3.55E-02	40	39.82 ± 0.14	0.05

Table S2 List of occupancy models and model fit test results of the posterior Predictive Loss (PPLC) and the Watanabe-Akaike Information criterions (WAIC) computed with the eDNAOccupancy R package (Dorazio and Erickson 2018). Models were run considering only *A. astaci* detections above LOD as positive (crayfish _eDNA above LOD) and including *A. astac*i detections below LOD (crayfish_eDNA below LOD). Aa_prevalence = *A. astaci* prevalence in crayfish population, waterbody = type of waterbody (lake, pond, river), nr_infected = number of infected crayfish per population, max_agent_lvl = maximum *A. astaci* agent level in crayfish tissue per population, altitude = altitude (metres above sea level) of the sampling site.

crayfish_eDNA above LOD	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC
(Ψ(.)θ(.)p(.))	11.3481	0.1482278	0.3140316	0.3662854	0.7262851	0.00000	0.000000
(Ψ(Aa_prevalence)θ(.)p(.))	11.4062	0.1491707		0.3629703	0.7274641	0.05810	0.000943
(Ψ(.)θ(Aa_prevalence)p(.))	11.8609	0.1589326	0.4819739		0.7177207	0.51280	0.010705
(Ψ(waterbody)θ(.)p(.))	11.2495	0.1481818	0.3265676		0.7271599	-0.09860	-0.000046
(Ψ(nr_infected)θ(.)p(.))	11.4361	0.1487926		0.3631071	0.7264827	0.08800	0.000565
(Ψ(.)θ(nr_infected)p(.))	11.9431	0.1585952	0.5063083		0.7213754	0.59500	0.010367
(Ψ(max_agent_lvl)θ(.)p(.))	11.3065	0.147856		0.3431814	0.7285686	-0.04160	-0.000372
(Ψ(altitude)θ(.)p(.))	11.3932	0.1499069		0.3295991	0.7282436	0.04510	0.001679
(Ψ(.)θ(altitude)p(.))	11.6261	0.1559487	0.3299644		0.7218359	0.27800	0.007721

crayfish_eDNA below LOD	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC
(Ψ(.)θ(.)p(.))	12.0071	0.1862969	0.5442907	0.7603092	0.9395317	0.00000	0.00000
(Ψ(Aa_prevalence)θ(.)p(.))	12.0106	0.1867358		0.7564865	0.9397353	0.00350	0.00044
(Ψ(.)θ(Aa_prevalence)p(.))	11.9326	0.186348	0.5474512		0.9398598	-0.07450	0.00005
(Ψ(.)θ(waterbody)p(.))	11.9494	0.187871	0.5938978		0.9394777	-0.05770	0.00157
(Ψ(waterbody)θ(.)p(.))	11.9832	0.1874039		0.7568805	0.9390969	-0.02390	0.00111
(Ψ(nr_infected)θ(.)p(.))	11.9535	0.1857738		0.7551305	0.9397412	-0.05360	-0.00052
(Ψ(.)θ(nr_infected)p(.))	11.9996	0.1879993	0.5472415		0.939956	-0.00750	0.00170
(Ψ(max_agent_lvl)θ(.)p(.))	12.0139	0.1865199		0.7556151	0.9393786	0.00680	0.00022
(Ψ(altitude)θ(.)p(.))	12.0034	0.1857521		0.7575291	0.9394368	-0.00370	-0.00054
(Ψ(.)θ(altitude)p(.))	12.0081	0.1868095	0.5446963		0.9392071	0.00100	0.00051

References

Dorazio RM, Erickson RA (2018) ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380. https://doi.org/10.1111/1755-0998.12735

Chapter 3

A survey of multiple aquatic wildlife pathogens of concern in Switzerland using an eDNA-based method for detection in water

Natalie Sieber[1,2], Alex King[3], Raphael Krieg[3], Armin Zenker[3], Christoph Vorburger[1,2], Hanna Hartikainen[1,2,4]

[1] Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

[2] ETH Zürich, Institute of Integrative Biology (IBZ), 8092 Zürich, Switzerland

[3] University of Applied Sciences and Arts Northwestern Switzerland, 4132 Muttenz, Switzerland

[4] University of Nottingham, School of Life Sciences, Nottingham, UK

In preparation

Abstract

Multiple pathogens infect and cause disease in aquatic wildlife and in species used in aquaculture. Conventional disease monitoring methods require laborious, costly and invasive capture and examination of host species, separately for every disease of interest. Pathogen detection techniques based on environmental DNA could in theory provide simultaneous surveys of multiple emergent aquatic pathogens across different host taxa. We conducted a survey investigating the occurrence of the crayfish pathogen *Aphanomyces astaci*, the amphibian pathogen *Batrachochytrium dendrobatidis*, and the fish pathogens *Saprolegnia parasitica* and *Tetracapsuloides bryosalmonae* in 280 sites in Switzerland. The presence of all four pathogens was investigated using eDNA-based methods in three replicate water samples on each site. Widespread distribution of *A. astaci*, *S. parasitica* and *T. bryosalmonae* was found, although *A. astaci* and *T. bryosalmonae* were not detected in some alpine river catchments. *B. dendrobatidis* was only found in five sites, since sampling locations did not include many amphibian breeding sites. Detection rates for all pathogens, and therefore co-detection, were higher in rivers than in lakes. Effects of several parameters on pathogen detection and DNA concentrations in water samples were investigated and discussed: elevation, lake surface area or river Strahler order, river slope and ecosystem integrity based on morphological criteria, as well as invertebrate and fish community composition. Detection of *T. bryosalmonae* in water samples matched previous fish infection data, though detection by eDNA was lower. PCR inhibition was rarely observed in water samples. Our study illustrates how eDNA-based techniques can be applied to monitor several pathogen species concurrently, indicating great potential for comprehensive disease monitoring schemes encompassing hosts and their pathogens across taxa.

Keywords: *Aphanomyces astaci*, *Batrachochytrium dendrobatidis*, environmental DNA, disease surveillance, pathogen monitoring, *Saprolegnia parasitica*, *Tetracapsuloides bryosalmonae*

Introduction

The last decades have seen the rise of a suite of emerging infectious diseases in wildlife, including aquatic species (Daszak et al. 2000). The emergence was often facilitated by man-made causes, such as climatic changes (Mitchell et al. 2005, Marcogliese 2008) and species introductions (Peeler et al. 2011). The mitigation and prevention of negative impacts of these diseases is of both conservational and economic interest and can only be achieved with effective management measures. These require comprehensive knowledge of disease agent occurrence and abundance gained by implementation of regular and comprehensive monitoring campaigns. However, conventional monitoring methods focus on laborious and costly capture of host species and their subsequent examination for infection, which is often invasive to the host. For example, electrofishing is used for capturing brown trout (*Salmo trutta)* whose kidneys are extracted and examined for *Tetracapsuloides bryosalmonae* infections causing proliferative kidney disease (PKD; Wahli et al. 2007). Conventional disease surveys are also often limited to host species of the same taxonomic group, such as fish or amphibians, and therefore pathogens infecting the same group. Thus, conventional methods are not feasible for surveys with a goal to provide a comprehensive picture of the distribution of multiple emergent aquatic pathogens across host taxonomic boundaries.

An opportunity for a non-invasive and less cost- and labour-intensive method for detecting multiple pathogens directly in their environment is presented by the growing field of environmental DNA. Environmental DNA (eDNA) refers to DNA acquired from the target organism's environment (e.g. water or soil) and not directly from the target itself, e.g. from tissue or blood samples (Thomsen & Willerslev 2015). Species detection from eDNA of in water has been successfully applied to a broad range of taxa such as fish (Adrian-Kalchhauser & Burkhardt-Holm 2016), amphibians (Biggs et al. 2015), crustaceans (Harper et al. 2018), invertebrates (Mächler et al. 2014), insects (Doi et al. 2017), plants (Anglès d'Auriac et al. 2019) and more. Pathogens with waterborne life stages can be detected using the same methods, where the detection likely targets stages such as free-swimming zoospores or eggs (pathogen eDNA sensu Pawlowski et al. 2020). While the term environmental DNA and its associated methods have been gaining attention for about two decades, emerging in the early 2000s (e.g. Rondon et al. 2000, Thomsen et al. 2012 for a short review), the concept of pathogen detection in water using molecular tools has been developed already in the early nineties (e.g. Toranzos et al. 1993) and focused mostly on human waterborne pathogens (Aw

& Rose 2012). Rapid advancements in molecular analytical methods have led to the fast development of the field (Bonadonna et al. 2019), and more studies have investigated detection of wildlife pathogens. Studies have mostly focused on pathogens held at least partly responsible for population declines of aquatic species of relevance for fisheries and conservation, such as several salmonid disease agents *Ceratonova shasta* (Hallett & Bartholomew 2006), *Gyrodactylus salaris* (Rusch et al. 2018) and *Myxobolus cerebralis* (Richey et al. 2018), including the PKD agent *T. bryosalmonae* (Fontes et al. 2017) and shellfish pathogen *Perkinsus marinus* (Audemard et al. 2004). By omitting the need to capture the host to find the pathogen, direct detection in water significantly decreases the effort and costs of disease agent monitoring. Methods are also more easily adaptable for detection of multiple pathogens of different host species, since instead of specialised procedures for capture and histopathological examination, DNA extraction and PCR methods follow the same, or similar, procedures for several target species. Simultaneous eDNA-based detection methods of multiple pathogen species could therefore help establish monitoring schemes that provide comprehensive and up-to-date knowledge of disease occurrence and co-occurrence. The thus garnered information facilitates the development of disease risk maps, including pathogen interactions, which help to apply informed management decisions.

One of the most ecologically important diseases is the crayfish plague, caused by the oomycete *Aphanomyces astaci*, which is largely responsible for population collapses of native European crayfish species (Holdich et al. 2009). It was brought to Europe unintentionally via ballast waters of trade ships and the release of North American crayfish species (Holdich 2003, Bohman et al. 2006) which have become invasive in Europe. While European crayfish species are highly susceptible to the disease, the invasive species act as asymptomatic carriers and reservoir species of the pathogen, due to their strong defense mechanisms (Nyhlén & Unestam 1980, Alderman et al. 1987). Another disease whose spread was facilitated by anthropogenic introduction into new areas through animal trade is chytridiomycosis in amphibians (Kilpatrick et al. 2010), which is caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Longcore et al. 1999). This pathogen can infect a large range of amphibians, with varying degrees of disease severity, from no clinical signs of infection (Daszak et al. 2004) to mass mortalities (Lips et al. 2006). It is considered the cause of decline or extinction of many amphibian species worldwide (Cunningham et al. 2017). Temperature-dependent proliferative kidney disease (PKD) caused by

Tetracapsuloides bryosalmonae (Phylum Cnidaria, Subphylum Myxozoa), severely impacts natural salmonid stocks as well as fish farms (Okamura et al. 2011) and is widespread in North America and Europe. The "water mould" *Saprolegnia parasitica*, an oomycete pathogen, occurs in all freshwater habitats around the world and can cause significant economic losses in aquaculture settings (van West 2006). Outbreaks due to highly virulent strains of *S. parasitica* have been also observed in wild populations (Paul & Belbahri 2012). These four pathogens have shown severe negative impacts in host populations and their close monitoring is therefore of great interest. The direct detection in water using eDNA-based techniques creates opportunity for conducting surveys for all four pathogens simultaneously, despite the range of hosts species they affect.

Detection in water has been applied for amphibian chytrid fungus *Batrachochytrium dendrobatidis* (Kirshtein et al. 2007) and crayfish plague *Aphanomyces astaci* (Strand et al. 2011), which has garnered substantial attention due to the severity of both diseases, leading to further studies applying the methods (Hyman & Collins 2012, Strand et al. 2014, Kamoroff & Goldberg 2017, Wittwer et al. 2018, Barnes et al. 2020). Crayfish plague has been monitored using eDNA-based methods in Norway for several years now, contributing to the 3Rs (replacement, reduction, refinement) of animal experiments (Strand et al. 2019a). Detection of *A. astaci* has been tested in large lakes (Strand et al. 2014) and streams (Wittwer et al. 2019) and for *B. dendrobatidis* in ponds (Walker et al. 2007, Hyman & Collins 2012), wetlands (Chestnut et al. 2014) and lakes (Kamoroff & Goldberg 2017). Assays for detection of PKD agent *T. bryosalmonae* in water were developed and applied by Fontes et al. (2017), Carraro et al. (2018) and Hutchins et al. (2018). *S. parasitica* was detected in water in the Loue river whose brown trout (*S. trutta*) and grayling (*Thymallus thymallus*) were heavily impacted by the pathogen (Rocchi et al. 2017).

The aim of this study was to map of the occurrence of *A. astaci*, *B. dendrobatidis*, *S. parasitica* and *T. bryosalmonae*, in Switzerland using a method of direct detection in water. The four pathogens have had severe impacts in Switzerland, as described above, except for *B. dendrobatidis*, which, while being present in many amphibian populations, has not been noted to have caused mass mortalities (Tobler et al. 2012). We used an eDNA-based method developed and tested in lab experiments (Chapter 1) and in the field (Chapter 2). The method involves *in situ* filtration of large water volumes and species-specific quantitative real-time PCR (qPCR) assays. Therefore, presence of all the four pathogens was analysed by qPCR in the same water samples collected throughout Switzerland. In addition to occurrence of the

agents, we were interested in patterns of detection rates and disease agent DNA concentrations in water according to types of waterbodies, elevation of sampling sites, lake surface area, Strahler order for rivers, river slope, river ecosystem integrity criteria and in the co-occurrence of several pathogens at the same site. Furthermore, we investigated inhibition in water samples as a potential factor influencing detection and DNA concentrations of the targeted disease agents.

Methods

Study sites and eDNA sampling

Sites of the survey were chosen to cover major waterways and lakes throughout Switzerland. Additional sites of interest were chosen by the cantonal governments providing funding to this project, resulting in 280 sites, 212 running, and 68 standing water (Fig. 1 and see Table S1 in the Supplement for site details). The majority of sites are situated in the Swiss midlands, fewer sites were sampled in montane areas due to lower presence of the targeted pathogens in those areas. Sampling was conducted from May to September in 2017 and from May to October in 2018 by two teams of two people each. Water samples were collected using an in-situ filtration system described in Chapter 1, further adapted to field sampling as in Chapter 2. Briefly, three 5 L water samples were taken per site and in case of clogging of a filter before reaching 5 L, up to six filters were collected, and a negative control using clean MilliQ water was taken before sampling at each site. The tubes containing the eDNA filters were stored on ice or in a dry shipper, which was cooled with liquid nitrogen before the trip, during multi-day trips (cantons Graubünden and Ticino), and subsequently stored at -80°C until extraction.

Environmental DNA extraction and real-time quantitative PCR

The eDNA extraction work was conducted in two separate labs in dedicated pre-PCR rooms (EAWAG in Dübendorf and FHNW in Muttenz). The DNEasy Power Water kit (Qiagen AG, Hombrechtikon, Switzerland) was applied using procedures described in Chapter 1 (Text S2 in the Supplement). No-template controls were included in all extraction runs and extracted samples stored at -20°C until further analysis.

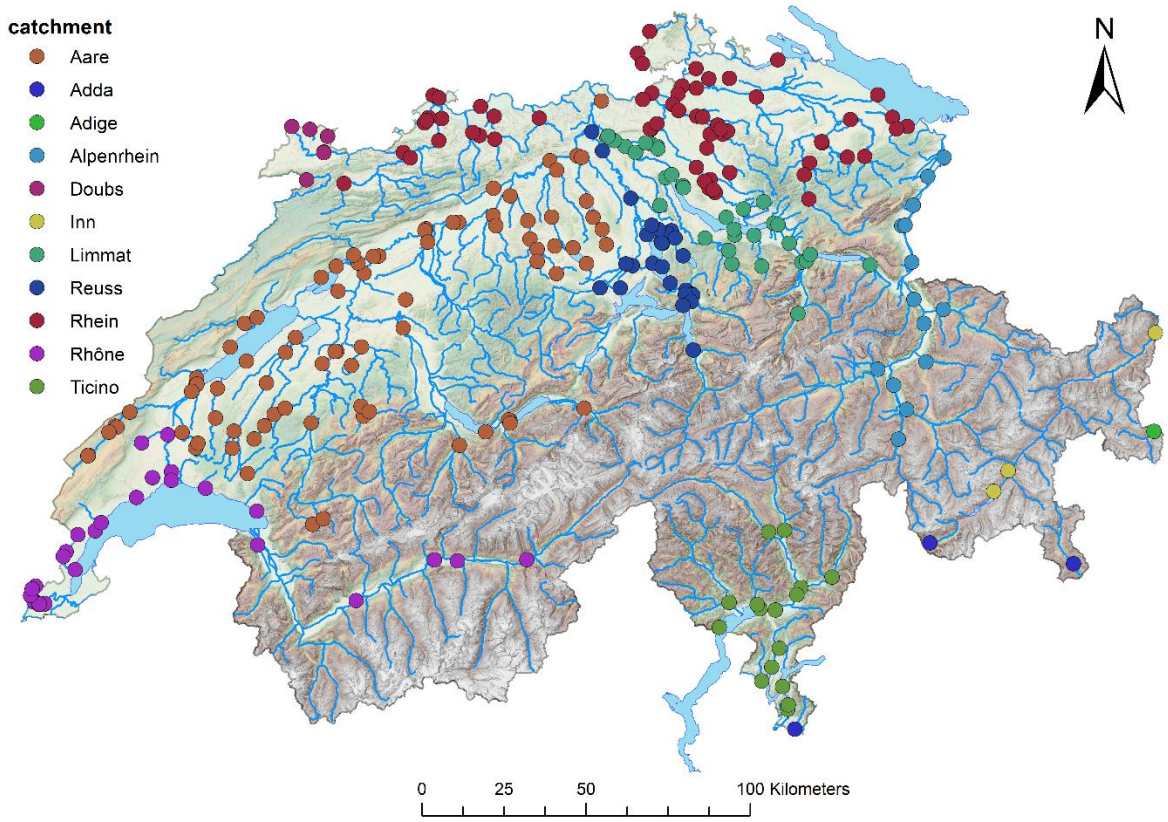


Figure 1. Map of Switzerland with sampling sites colour-coded according to catchment area.

All water samples were analysed for all four pathogen species in separate reactions using probe-based Taqman real-time quantitative PCR (qPCR) on a LightCycler 480 (Roche, Basel, Switzerland). Triplicate reactions were set up using a QIAgility pipetting robot (Qiagen AG, Hombrechtikon, Switzerland) and negative controls were included in each run. The samples were tested in separate runs for different pathogen species. An overview of the assays and their original references are listed in Table 2 of the general introduction of the thesis. Reaction setup and thermal cycling for a *A. astaci* are described in Chapter 2 and for *B. dendrobatidis* and *T. bryosalmonae* in Chapter 1. Implementing the assay developed by Rocchi et al. (2017) targeting the ITS1 and ITS2 regions, reactions for *S. parasitica* contained 5 µl of LightCycler 480 Probes Master buffer (Roche, Basel, Switzerland), forward primer Primer-F and reverse primer Primer-R each at concentration of 900 nM, the probe Probe-R at 200 nM and 2.5 µl of template DNA. Thermal cycling for *S. parasitica* included an initial ten minutes at 95°C and 45 cycles of 15 seconds at 95°C followed by 60 seconds at 60°C. Finally, a 10 second cooling step at 40°C was implemented. In addition to the pathogen assays, and to test for inhibition, an internal positive control (IPC) was spiked into each eDNA sample in a separate qPCR run (see Chapter 1 for detailed procedures).

To quantify the DNA content of samples and to determine the limit of detection (LOD) and quantification (LOQ) for each assay we used a double-stranded Gblocks fragment (Integrated DNA Technologies) containing target sequences of all pathogen assays (see Supplement Text S1). LOD and LOQ for *A. astaci* are reported in Chapter 2 and for *B. dendrobatidis* and *T. bryosalmonae* in Chapter 1. For *S. parasitica*, the LOD was determined to be at Cq-value of 35.752 and the LOQ at a concentration of 22.2 copies μl^{-1} using the same procedures as in Chapter 1 and 2 (see Table S2 in the Supplement for dilution series results). The standard curves of all four pathogens used for definition of the LOD and LOQ are visualised in Figure S1 in the Supplement.

Data analysis

The qPCR raw output data was processed as described in Chapter 1. Disease agent DNA concentration of a water sample was defined as above LOD if the DNA concentration in one or more of the qPCR replicates exceeded the LOD. Detection at the site level was considered successful if at least one water sample of a site was positive for pathogen DNA. Mean DNA concentrations per water sample were calculated with positive qPCR replicate values. Mean DNA concentrations per site were calculated from mean sample concentrations. All analyses were conducted in R version 3.6.1. (R Core Team 2019). Eleven major watersheds were defined, in alphabetic order: Aare, Adda, Adige, Alpenrhein, Doubs, Inn, Limmat, Reuss, Rhein, Rhône and Ticino (Fig. 1). Sampled river stretches were further assigned into flat, semi-steep and steep slope categories according to Swiss river typology categories (BAFU 2015). Three different categories for ranking river stretches for their natural state, or ecosystem integrity, collected and ranked by the Federal Office for the Environment (Kunz et al. 2016) were integrated in the analysis. The three rankings were "Ökomorphologie" (ecomorphology), ranking morphological aspects of the river (BAFU 1998), "Makrozoobenthos", ranking integrity based on invertebrate community structure (BAFU 2019), and lastly, fish community structure. The fish community ranking is based on presence of indicator species, fish abundance and frequency of deformities in fish (BAFU 2004). All rankings included four categories, from 1 = unnatural to 4 = natural. The effect of waterbody type, i.e. lake or river, sampling site elevation (m.a.s.l.), lake surface area, river Strahler order, slope and river ecosystem integrity criteria (ecomorphology, invertebrate and fish community composition) on pathogen detection was investigated using binomial generalised linear mixed effects models with watershed as random factor.

Pathogen DNA concentration estimates in water were transformed using Tukey's transformation for analysis (Tukey 1977). We then applied linear mixed effects models to test the effects of waterbody type and elevation, lake surface area, river Strahler order, slope and the three ecosystem integrity criteria on mean DNA concentrations per survey site. Since lake surface area and Strahler order are only defined for lakes and rivers, respectively, they were tested separately from waterbody type. Also, waterbody type was not analysed for *B. dendrobatidis* DNA concentrations because *B. dendrobatidis* was only found in rivers. Further, since the three ecosystem integrity criteria were not available for all sampled sites, they were analysed separately. The same analyses were also conducted including DNA concentrations above LOQ only. *T. bryosalmonae* DNA concentrations above LOQ were only detected in rivers and therefore not analysed with waterbody type and lake surface area. Association between DNA concentrations of two disease agent species in the same water sample were examined using linear mixed models with survey site as random factor for each pathogen pairing, except for the pairing *B. dendrobatidis* and *T. bryosalmonae*. For that pairing, a linear model was implemented without random effect, because only single samples from sampling sites had concentrations for both agents. Effects of waterbody type, elevation, lake surface area, river Strahler order, slope and ecosystem integrity criteria on the number of pathogens co-detected per site (no detections to all four detected: 0 – 4) were analysed using binomial generalised linear mixed effects models with watershed as random factor. Co-detection between pathogen pairs was investigated with Pearson's Chi-squared tests with Yates' continuity correction for each agent pairing. Inhibition in water samples was measured as described in Chapter 1 using an internal positive control.

Hierarchical occupancy models were run according to Dorazio and Erickson (2018) to calculate occupancy probabilities on the level of sampling site, water sample and qPCR replicate for each pathogen except *B. dendrobatidis*, which was excluded from this analysis due to very low detection rates. All models were run with 11'000 MCMC iterations and effect of elevation, waterbody type, river Strahler order and lake surface area were analysed by model selection using PPLC (Gelfand & Ghosh 1998) and WAIC (Watanabe 2010) criterions. Furthermore, we used the equation $1 - (1 - \theta)^n \geq 0.95$ to determine the number of water samples (n) required per sampling site to reach detection success of 95% or above for estimated pathogen DNA detection probabilites per water sample (θ).

We further compared *T. bryosalmonae* detection data with PKD prevalence data from fish collected in previous studies (Wahli et al. 2008). Fish were not collected at the same sites, but 79 sites were deemed close enough to our sampling sites for comparison. Detection rates

were compared with a Pearson's Chi-squared tests with Yates' continuity correction and a McNemar's Chi-squared test with continuity correction.

We used binomial generalised linear mixed effects models to investigate the effect of waterbody type, elevation, lake surface area, river Strahler order and slope on qPCR inhibition in water. The effect of inhibition on detection rates in water samples was analysed for each disease agent with Pearson's Chi-squared tests with Yates' continuity correction. Further, influence of inhibition on disease agent concentrations in water samples were analysed with linear mixed effects models with survey site as random factor.

Results

A preliminary report of this survey's findings regarding the occurrence of the four targeted disease agents in Switzerland was published by Krieg et al. (2019) for the Swiss Federal Agency for the Environment. A total of 280 sites consisting of 68 lake and 212 river sites were sampled. *Aphanomyces astaci* was detected in 87 of 262 (33.21 %) sites (Fig. 2a), *B. dendrobatidis* in 5 out of 280 (0.02 %) sites (Fig. 2b), *S. parasitica* in 205 out of 278 (73.74 %) sites (Fig. 2c) and *T. bryosalmonae* in 59 out of 279 (21.15 %) sites (Fig. 2d). Results are further listed in Table S1 in the Supplement. For *A. astaci* and *S. parasitica* some sites were excluded due to amplification in the sampling negative controls (n = 18 for *A. astaci*, n = 2 for *S. parasitica* and n = 1 for *T. bryosalmonae*). *A. astaci* was found in all major watersheds except the Inn and Adige (Rom in Val Müstair) in the alpine region (Fig. 2a) and *B. dendrobatidis* was detected in single locations of the Adda, Alpenrhein, Limmat, Rhein and Ticino catchments (Fig. 2b). *S. parasitica* was present in all major catchments, while *T. bryosalmonae* was not found in the alpine Adda, Adige and Inn catchments (Fig. 2d). *A. astaci* (χ^2 = 5.773, df = 1, p = 0.016), *S. parasitica* (χ^2 = 23.650, df = 1, p < 0.001) and *T. bryosalmonae* (χ^2 = 9.815, df = 1, p = 0.002) were significantly more often detected in river than lake sites (*A. astaci*: n = 12 / 63 lake sites, n = 75 / 199 river sites; *S. parasitica*: n = 33 / 67 lake sites, n = 172 / 211 river sites; *T. bryosalmonae*: n = 1 / 68 lakes, n = 58 / 211 river sites). All five sites where *B. dendrobratidis* was detected were rivers, but due to the low detection rate, no clear pattern of detection between types of waterbodies was observed (χ^2 < 0.001, df = 1, p = 0.993). *A. astaci* and *T. bryosalmonae* were slightly more frequently detected at lower elevations, but the relationship was not significant in either case (χ^2 = 3.329, df = 1, p = 0.068 and χ^2 = 1.973, df = 1, p = 0.160, respectively). No altitudinal differences regarding detection were noted with *B. dendrobatidis* (χ^2 = 0.076, df = 1, p = 0.783) nor *S.*

parasitica (χ^2 = 0.670, df = 1, p = 0.413). *S. parasitica* detection increased with lake surface area (χ^2 = 14.668, df = 1, p < 0.001), which was not observed for *A. astaci* (χ^2 = 0.177, df = 1, p = 0.674) nor *T. bryosalmonae* (χ^2 = 0.200, df = 1, p = 0.655). Neither Strahler order, nor slope categories of river sections had a significant effect on the detection of any pathogens, nor was their detection probability related to any of the three river ecosystem integrity criteria, i.e. ecomorphology, invertebrate community and fish community composition (see Table S3 in the Supplement for analysis results).

Hierarchical occupancy models results with constant parameters on sampling site, water sample and qPCR replicate for *A. astaci*, *S. parasitica* and *T. bryosalmonae* are shown in Table 1. The inclusion of elevation, water type, river Strahler order and lake surface area did not improve model fit (see Table S4 in the Supplement for detailed results).

Table 1. Occupancy probabilities of sampling site Ψ(.), water sample θ(.) and qPCR replicate p(.), and number of water samples (n) required per sampling site to reach detection probabilities of ≥ 95% according to θ(.). Probability estimates are listed per pathogen species.

species	Ψ(.)	θ(.)	p(.)	# water samples (n)
Aphanomyces astaci	0.371	0.544	0.804	4
Saprolegnia parasitica	0.808	0.605	0.908	4
Tetracapsuloides bryosalmonae	0.247	0.490	0.810	5

Pathogen DNA concentrations in water samples

Only in *S. parasitica* did mean DNA concentrations in water samples decrease with increasing elevation (r = -0.272, χ^2 = 9.807, df = 1, p = 0.002), which, however, was not observed for *A. astaci* (r = -0.104, χ^2 = 1.158, df = 1, p = 0.305), *B. dendrobatidis* (r = -0.232, χ^2 = 0.741, df = 1, p = 0.390) nor *T. bryosalmonae* (r = -0.172, χ^2 = 2.090, df = 1, p = 0.148). There were no significant differences of DNA concentrations of any pathogens between lakes and rivers, neither was there an effect on concentrations by lake surface, nor by river Strahler order, slope categories and ecosystem integrity criteria (see Table S5 in the Supplement for test results).

When only pathogen DNA concentrations above the limit of quantification (LOQ) were included, negative elevation-dependent DNA concentrations of *T. bryosalmonae* (r = -0.356, χ^2 = 5.171, df = 1, p = 0.023) were observed in addition to *S. parasitica* (r = -0.186, χ^2 = 4.048, df = 1, p = 0.044), while no such effect was observed for *A. astaci* (r = -0.296, χ^2 = 3.195, df = 1, p = 0.074). Moreover, *A. astaci* DNA concentrations above LOQ increased

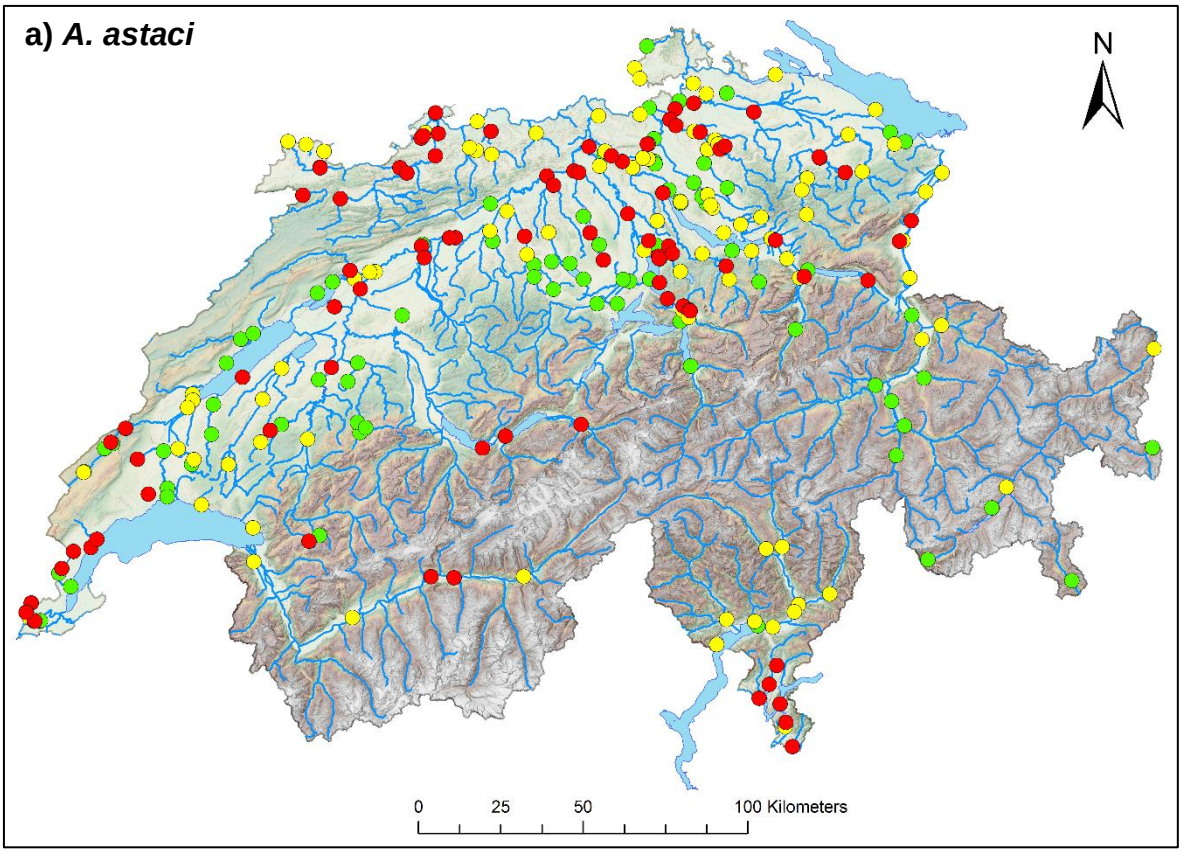
with lake surface area ($r = 0.803$, $\chi^2 = 20.773$, $df = 1$, $p < 0.001$) and *S. parasitica* DNA concentrations above LOQ decreased with river order ($r = -0.134$, $\chi^2 = 4.246$, $df = 1$, $p = 0.039$). River slope was not observed to influence DNA concentrations above LOQ for any of the pathogens, nor were any of the river ecosystem integrity criteria, i.e. ecomorphology invertebrate and fish community (see Supplement Table S6 for test results).

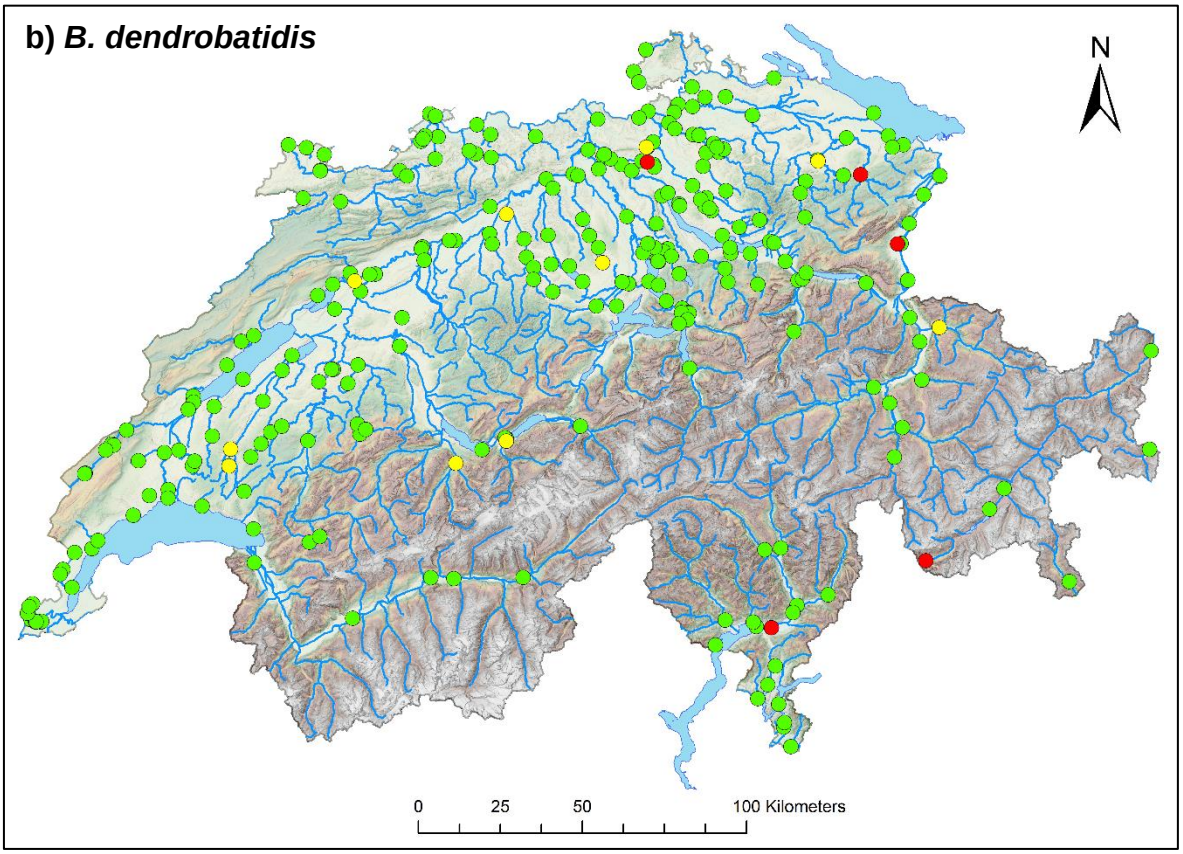
DNA concentrations in water samples positively correlated between *S. parasitica* and *T. bryosalmonae* ($r = 0.355$, $\chi^2 = 19.209$, $df = 1$, $p < 0.001$), between *S. parasitica* and *A. astaci* ($r = 0.103$, $\chi^2 = 22.591$, $df = 1$, $p < 0.001$), *S. parasitica* and *B. dendrobatidis* ($r = 0.729$, $\chi^2 = 22.294$, $df = 1$, $p < 0.001$), and to a lesser degree between *A. astaci* and *B. dendrobatidis* ($r = 0.025$, $\chi^2 = 4.053$, $df = 1$, $p = 0.044$). No other associations between disease agent DNA concentrations in water samples were found (*A. astaci – T. bryosalmonae*: $r = 0.132$, $\chi^2 = 1.238$, $df = 1$, $p = 0.266$; *B. dendrobatidis – T. bryosalmonae*: $r = 0.462$, $F_{1,4} = 1.181$, $p = 0.338$).

Patterns of co-detection

All four infectious agents were detected at one site (river Simmi near Gams) and all except *B. dendrobatidis* in 29 sites and all except *A. astaci* in two sites. In 70 sites, two agents were detected. Co-detection was highest in rivers ($\chi^2 = 33.860$, $df = 1$, $p < 0.001$). In rivers, most co-detections were observed in sites with semi-steep slopes ($\chi^2 = 15.707$, $df = 2$, $p < 0.001$). Elevation, lake surface area, river Strahler order and ecosystem integrity criteria (morphology, invertebrate and fish community) did not show significant association with number of co-detected pathogens ($\chi^2 = 2.321$, $df = 1$, $p = 0.128$; $\chi^2 = 0.094$, $df = 1$, $p = 0.760$; $\chi^2 = 0.276$, $df = 1$, $p = 0.599$; $\chi^2 = 0.210$, $df = 1$, $p = 0.647$, $\chi^2 = 0.084$, $df = 1$, $p = 0.773$ and $\chi^2 = 0.249$, $df = 1$, $p = 0.618$, respectively). Detection of *A. astaci* and *S. parasitica*, *A. astaci* and *T. bryosalmonae*, and *S. parasitica* and *T. bryosalmonae* correlated ($\chi^2 = 12.488$, $df = 1$, $p < 0.001$, $\chi^2 = 17.736$, $df = 1$, $p < 0.001$, and $\chi^2 = 14.643$, $df = 1$, $p < 0.001$, respectively). *B. dendrobatidis* detection did not correlate with detection of any of the other disease agents (*A. astaci*: $\chi^2 = 0.017$, $df = 1$, $p = 0.898$, *S. parasitica*: $\chi^2 = 0.516$, $df = 1$, $p = 0.473$, *T. bryosalmonae*: $\chi^2 = 2.569$, $df = 1$, $p = 0.109$). Pairwise co-detection of *A. astaci* with *S. parasitica* and of *A. astaci* with *T. bryosalmonae* did not show significant patterns with any sampling site properties (see Supplementary Table S7 for test results). Pairwise co-detection of *S. parasitica* with *T. bryosalmonae* decreased with elevation ($\chi^2 = 4.164$, $df = 1$, $p = 0.041$)

and a similar, but insignificant trend was observed for pairwise detection of *A. astaci* with *T. bryosalmonae* ($\chi^2 = 2.912$, df = 1, p = 0.088).





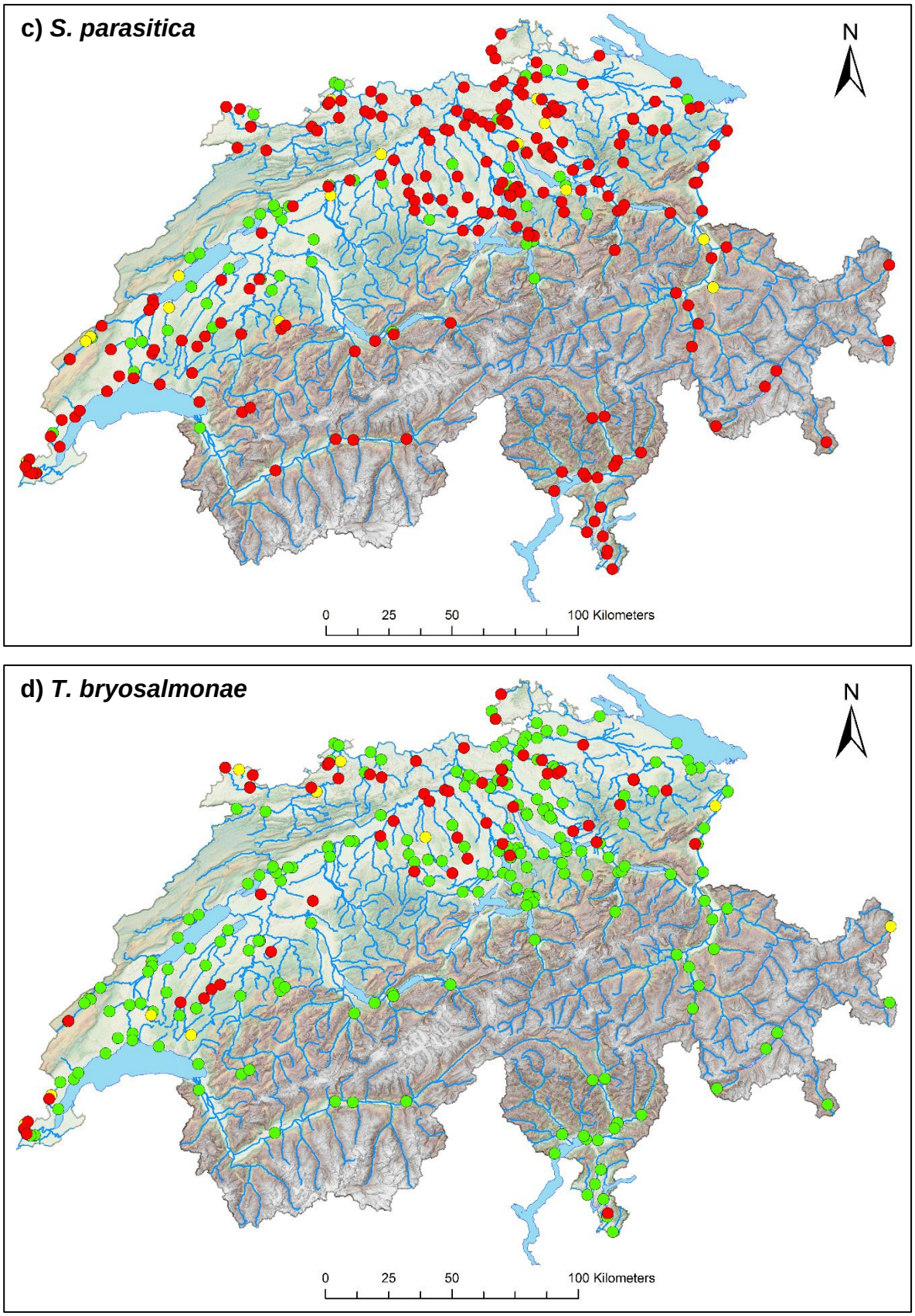


Figure 2. Distribution maps in Switzerland of a) *A. astaci*, b) *B. dendrobatidis*, c) *S. parasitica* and d) *T. bryosalmonae*. Red = positive detection, yellow = uncertain detection below limit of detection (LOD), green = negative detection.

Comparison of *T. bryosalmonae* DNA detection in water to PKD infection data of fish

T. broysalmonae was detected in both water samples and fish in 25 of the 79 river sites were both datasets were available (see Table S8 in the Supplement for site information and detection results). In 27 sites, neither methods detected the parasite. However, PKD-infected fish were captured from 26 sites where water samples did not detect the disease agent in water and only in one site *T. bryosalmonae* DNA was detected in water, but not in fish. While results of the two methods were associated ($\chi^2 = 14.914$, df = 1, $p < 0.001$), *T. bryosalmonae* was more successfully detected in fish (McNemar's $\chi^2 = 21.333$, df = 1, $p < 0.001$) in the 79 analysed river sites.

Inhibition of water samples

Only 2.37 % (n = 21 / 887) of the eDNA samples from 13 sites (total sites analysed n = 266) showed signs of inhibition, i.e. had IPC Δ Cq-values ≥ 3. The 13 inhibited sites included nine rivers and four lakes, but this difference was not significant ($\chi^2 = 0.059$, df = 1, $p = 0.808$). Further, neither elevation of a sampling site influenced probability of inhibited water samples ($\chi^2 = 0.151$, df = 1, $p = 0.698$), nor did lake surface area ($\chi^2 = 0.744$, df = 1, $p = 0.389$), nor river Strahler order ($\chi^2 = 0.195$, df = 1, $p = 0.659$) nor slope ($\chi^2 = 0.757$, df = 1, $p = 0.685$). Inhibition affected detection rates of *S. parasitica* ($\chi^2 = 14.59$, df = 1, $p < 0.001$), in that the oomycete was detected only in one of 21 samples with IPC Δ Cq-value ≥ 3, while *S. parasitica* detection rate in uninhibited samples, i.e. with IPC Δ Cq-values < 3, was almost 50 % (n = 424 / 859). Detection was not significantly influenced by inhibition for *A. astaci* ($\chi^2 = 0.1$, df = 1, $p = 0.752$), *B. dendrobatidis* ($\chi^2 < 0.001$, df = 1, $p = 1$) and *T. bryosalmonae* ($\chi^2 = 1.701$, df = 1, $p = 0.192$). Higher IPC Δ Cq-values were associated with lower DNA concentration estimates (copies μl^{-1}) of *S. parasitica* (r = -0.176, SE = 0.02, t = -4.937, $p < 0.001$) and *T. bryosalmonae* (r = -0.339, SE = 0.038, t = -2.079, p = 0.040). No association between IPC Δ Cq-values and DNA concentrations was observed for *A. astaci* (r = -0.043, SE < 0.001, t = 0.023, p = 0.981) and *B. dendrobatidis* (r = -0.394, SE = 0.323, t = -1.152, p = 0.267).

Discussion

Investigations of parasite occurrence within taxonomically different host groups that share the same habitat can inform on the environmental drivers and interactions that govern patterns of parasite community assembly. Ultimately, understanding pathogen coexistence may lead to better disease management strategies that benefit host communities at the ecosystem level. Developing such measures is challenged by difficulties in monitoring, and we provide the first survey encompassing pathogens of amphibians, fish and crayfish using the same water samples. We surveyed a total of 280 sites for the occurrence of four pathogens, *A. astaci, B. dendrobatidis, S. parasitica* and *T. bryosalmonae,* using methods for detection in water and created maps of occurrence of Switzerland (Figs. 2a - 2d). We observed a very widespread distribution of *S. parasitica* and to a lesser extent, of *A. astaci*. *T. bryosalmonae* was mostly detected in the Swiss midlands. Detections of any of the target pathogens were absent or very rare in the alpine catchments (Adige, Adda and Inn), likely reflecting parasite distribution patterns, although sampling effort was also lower in these regions. Detection of *B. dendrobatidis* was too rare to create an informative map of occurrence, presumably due to majority of the sampling sites not reflecting the preferred habitat for infected hosts of this pathogen. Given the previous knowledge on the occurrence of *A. astaci* (Jean-Richard 2013) and *T. bryosalmonae* (Wahli et al. 2008), and the known ubiquity of *S. parasitica* in freshwater systems, we deem the survey successful in providing distribution data for those three pathogens, but not for *B. dendrobatidis.* In the following sections we discuss the results for all pathogens in turn.

Aphanomyces astaci distribution

Aphanomyces astaci was detected in third of all surveyed sites (87 of 262 sites) and appears to be present in all major Swiss watersheds. Exceptions were the upper reaches of the Rhein catchment, the Inn and upper Ticino catchments and the mountain valleys of Müstair and Poschiavo (Adige and Adda catchments), where low water temperatures likely preclude the occurrence of any crayfish. However, below LOD signals from several sites of the upper Ticino catchment suggest a potential presence of the pathogen (see below), possibly in warmer tributaries to the main river (Fig. 2a). The invasive North American crayfish which act as carriers of the pathogen and are present in all major waterways and lakes in Switzerland (Stucki & Zaugg 2011), and likely contribute to the widespread detection of *A. astaci*. The last *A. astaci* survey in Switzerland was conducted in 2012 and comprised 55 sites (Jean-

Richard 2013). For detection, invasive crayfish populations were sampled and crayfish tissues analysed for *A. astaci* by quantitative PCR. Despite the smaller number of analysed sites, a similarly widespread distribution of *A. astaci* was observed.

A substantial number of sites showed *A. astaci* detections in estimated concentrations below the LOD. Interpretation of such low-level detections is challenging since weak amplification due to low target DNA content can be difficult to distinguish from unspecific amplification. The assay for *A. astaci* used in this study (Vrålstad et al. 2009) has amplified a closely related *Aphanomyces* species before (Kozubíková et al. 2009, Viljamaa-Dirks & Heinikainen 2019). This issue had prompted changes in the thermal cycling regime (Kozubíková et al. 2011, Strand et al. 2014), which we followed here, at the price of decreasing the sensitivity of the assay. Therefore, it is possible that the detections below LOD observed in this study may indicate low levels of *A. astaci* presence. These patterns could be confirmed using a more sensitive and specific assay combined with sequencing of the PCR product. In Chapter 2 we observed below LOD signals in water from sites where *A. astaci* indeed occurred in crayfish at low prevalence, indicating that below LOD detections of this harmful pathogen should be investigated further as signals of potential occurrence. Therefore, we suggest taking more water and potentially crayfish samples, to obtain a better understanding of disease risk in the area.

Batrachochytrium dendrobatidis distribution

Detection rate of *Batrachochytrium dendrobatidis* was low in this survey, with only 5 sites out of 280 with positive detection (Fig. 2b). This result underlines the significance of the sampling design to capture target pathogens, but also the robustness of the qPCR assays against unspecific amplification and false positive results in environmental samples containing a complex mix of potential templates. Our sampling scheme mainly targeted main waterways for good overall coverage of Switzerland, i.e. it favored extent over detail, and it included many sites of interest to contributing Cantons, with a focus on fish and crayfish diseases This focus biased the survey against the detection of *B. dendrobatidis*, since amphibian species mostly occur in smaller streams and ponds, or show strong seasonal dynamics in their occurrence in larger waterbodies. For example, in Lake Lucerne, common toads (*Bufo bufo*) can be found from early spring to early summer but will mostly be absent for the remaining seasons after metamorphosis of the tadpoles (N. Sieber, personal observation). Previous studies with successful implementation of the eDNA-based detection

method for *B. dendrobatidis* applied more targeted approaches by sampling known and / or suitable amphibian habitats (Kirshtein et al. 2007, Walker et al. 2007, Chestnut et al. 2014, Kamoroff & Goldberg 2017, Mosher et al. 2018, Barnes et al. 2020) and also considered seasonal timing of sampling according to the amphibian life cycle (Schmidt et al. 2013). Most of these studies sampled waterbodies with high turbidity, such as wetlands and ponds, and filters were therefore prone to clogging, limiting the filtered water volume: volumes per filter in the mentioned studies varied from 20 ml to 2.4 L. We experienced similar limitations in this study when sampling ponds, with filters clogging as early as after 0.5 L had been filtered. Therefore, sampling schemes should consider and design the sample collection methods based on habitat requirements of the affected hosts. In ponds and other turbid water bodies, strategy focusing on maximising number rather than volume of water samples might be more appropriate, as suggested by Mosher et al. (2018). Furthermore, Chestnut et al. (2014) modelled the detection probability of *B. dendrobatidis* in water samples as a function of the number and volume of collected water samples and show that similar detection probabilities (> 95 %) can be reached with 60 ml samples compared to 600 ml samples if five instead of four samples are collected per site.

Saprolegnia parasitica distribution

Saprolegnia parasitica was detected in the majority of sites (205 of 278, Fig. 2c), which could be expected since it is known global distribution (van West 2006). The results show that *S. parasitica* is present country-wide, and the risk of disease depends on several factors. First, *S. parasitica* strain diversity is high in Switzerland (Ravasi et al. 2018) but virulence levels invoked by different strains are variable and largely unknown. Secondly, *S. parasitica* often causes secondary infections of wounded, immunocompromised or otherwise stressed fish (Neish 1977, Howe & Stehly 1998). Therefore, factors increasing *S. parasitica* outbreak risk could be manifold, from host activity, e.g. during mating season, to stressful environmental conditions, such as drought and increased water temperatures (Pickering & Willoughby 1982, van West 2006).

Tetracapsuloides bryosalmonae distribution

Tetracapsuloides bryosalmonae was found in 21.15 % of (59 of 279) sites and appears mostly in low elevations of the northern part of Switzerland (Fig. 2d). Previous knowledge about the occurrence of *T. bryosalmonae* in 287 sites throughout Switzerland was collected

from 2000 to 2006 in several electrofishing campaigns followed by histopathological examination of almost 7000 fish (Wahli et al. 2002, 2007, 2008). Of the 287 sites, 79 were close, or overlapped sampling sites in this study and *T. bryosalmonae* detection in water was compared to fish infection data (see Table S8 in the Supplement). *T. bryosalmonae* was detected in water in half the sites were infected fish were found. Reasons for this low detection in water compared to fish could be low pathogen spore loads in the river or their heterogeneous distribution as discussed in Chapter 1.

Effect of waterbody type and size and elevation on pathogen detection and DNA concentrations

Proportionally to the total number of sites, most detections of *A. astaci*, *S. parasitica* and *T. bryosalmonae* were observed in rivers. Host species such as crayfish and fish might appear in lower densities or just in certain areas of large waterbodies such as lakes, possibly rendering the disease agent eDNA distribution patchy and concentrations low. Also, *S. parasitica* concentrations above the LOQ decreased with river Strahler order, i.e. in larger rivers. Therefore, higher sampling effort, i.e. sampling several locations of a lake or a large river, might be needed to reach similar detection levels as smaller waterbodies. Arguing against the idea of an effect of dilution according to waterbody size is the higher detection rate *of S. parasitica* and higher *A. astaci* DNA concentrations above limit of quantification (LOQ) of water samples from larger lakes and the lack of influence of river size, approximated by Strahler order in this study, on disease agent detection. Also, no significant variation in disease agent DNA concentrations, including those below LOQ, relative to waterbody type and size was observed. However, this lack of a pattern in DNA concentrations could be rather due to low accuracy of the qPCR quantification, especially in low DNA content samples (Mauvisseau et al. 2019), than a missing dilution effect. Most water samples contained agent concentrations below the limit of quantification (*A.astaci*: 79.41 %, *B. dendrobatidis*: 100 %, *S. parasitica*: 38.62 %, *T. bryosalmonae*: 61.98 %), which is a common property of eDNA samples. Furthermore, the complexity and dynamic of the aquatic system could limit accuracy of eDNA quantification due to heterogeneous distribution of agent spores and therefore, pathogen DNA, in the system (see Chapter 1). Heterogeneity in the environment also leads to stochasticity in detection of the pathogen, which could be minimised with higher sampling effort (see Chapter 1). This is strongly suggested by the occupancy modelling results, which show that for *A. astaci* and *S.*

parasitica, four, and for *T. bryosalmonae* five water samples would need to be collected per site to reach detection rates of ≥ 95%, given that the pathogen is present.

Small altitudinal differences in detection were observed for *A. astaci* and *T. bryosalmonae* and are supported by absence of the two parasites from the high alpine Inn and Adige (Rom in Val Müstair) catchments. Crayfish do not commonly appear in montane and alpine regions due to temperature limitations (Chucholl 2017). Therefore, higher occurrence of *A. astaci* in lower elevations is expected. When considering the concentrations above LOQ, *T. bryosalmonae* DNA concentrations in water samples decreased with increasing elevation. Elevation correlates negatively with water temperatures and Wahli et al. (2008) have previously observed an elevation-dependent *T. bryosalmonae* occurrence in Switzerland. Infected trout populations are generally not found above elevations of 800 metres above sea-level. This study shows a similar pattern with only one detection of *T. bryosalmonae* above 800 m. a. s. l., the river Orbe at 1014 m. a. s. l., where infected fish were observed before (Wahli et al. 2008). *T. bryosalmonae* development is affected by temperature in both the bryozoan host (Tops et al. 2009) and fish host (Bailey et al. 2018) and could influence detection rates in water (Fontes et al. 2017). Further, the specific habitat requirements of the bryozoan host likely limit the distribution *T. bryosalmonae* and explain more frequent occurrence at lower elevations. The rare occurrence, or even absence, of *A. astaci* and *T. bryosalmonae* in alpine regions indicates the importance of high elevation refugia from disease agents, threatened by the changing climate. Most Swiss lakes and rivers lie within the temperature range of *Saprolegnia parasitica* (Kitancharoen et al. 1996). Saprolegniosis of fish is most often observed during winter, especially after fast water temperature drops that stress the host fish (Quiniou et al. 1998). This could explain the lack of association of *S. parasitica* detection rates with elevation, even though *S. parasitica* DNA concentrations decreased with elevation, and it could also indicate that winter would be a more appropriate timing for surveying *S. parasitica*.

No effect on parasite detection rates and DNA concentrations was observed of slope and all three ecosystem integrity criteria of river stretches. Due to the widespread distribution of *S. parasitica* and the capabilities of *T. bryosalmonae* hosts, e.g. *S. trutta*, to survive and move around in steep rivers no association was expected for those two parasites. Crayfish and amphibians, however, usually do not appear in steep rivers. Here, the lack of association with *A. astaci* detection and concentrations might be due to the low number of steep rivers among sampled sites (n = 9) and generally low detection rates of *B. dendrobatidis*. Lack of

association with ecosystem integrity criteria could stem from the low sample size, especially for fish (n = 23) and invertebrate (n = 47) community structure and age difference between the datasets, i.e. the data for the criteria was collected from 2011 to 2014 (Kunz et al. 2016).

Patterns of co-occurrence of pathogen DNA in water

Detection of *A. astaci*, *S. parasitica* and *T. bryosalmonae* were correlated and co-detection was highest in rivers. The latter is not surprising since most detections of those species were noted in rivers. *Saprolegnia parasitica* can infect both fish and crayfish (Edgerton et al. 2002). This could explain the co-occurrence when considering that host individuals weakened by infection of either *A. astaci* or *T. bryosalmonae* are more likely to be infected by the opportunistic *S. parasitica* (van den Berg et al. 2013). Also, coexistence of host species, i.e. crayfish and susceptible fish species, is likely and could be responsible for the correlated occurrence of disease agents. Less frequent appearance of *T. bryosalmonae* with *S. parasitica*, and to a lesser extent, *with A. astaci* in higher elevations could be due to low occurrence of bryozoans, crayfish and certain fish species in these areas. The analysis of water samples for host DNA would therefore be a valuable addition of eDNA surveys targeting parasites (Strand et al. 2019b) Such direct measures of host distribution may explain pathogen detection patterns better than the indirectly correlated elevation and river ecosystem integrity criteria, which here did not significantly affect co-detection of pathogens. However, pathogen and host occurrence and abundance might not be expected to correlate. For example, while hosts are most abundant in intact river ecosystems in elevations with adequate water temperatures, pathogens might be more abundant in sites where host populations are exposed to stressors, such as habitat degradation and temperatures near their thermal tolerance limits. Also, healthy host populations could be either free of infection or have low pathogen and infection prevalence leading to pathogen DNA concentrations in water below detection level with eDNA techniques. Therefore, a quantitative approach targeting all hosts in the pathogen life cycle, coupled with environmental parameters might be an exciting future avenue towards more comprehensive multiple disease agent and disease risk mapping to safeguard ecosystem health.

However, co-detection could also have been influenced by variability of DNA extraction efficiency introduced by the filter (see Chapter 1 for a detailed discussion). This implies that no pathogens could be detected in a water sample whose DNA extraction was not efficient, even though some of the pathogens could have been present in the water. While DNA

concentrations of *A. astaci* and *S. parasitica*, and *S. parasitica* and *T. bryosalmonae* in water samples correlated, quantification via qPCR was highly variable, making a quantitative association of disease agent DNA concentrations with potential host population health a challenging prospect. Comparative, i.e. between sampling sites, and qualitative associations have been described between disease agent DNA concentrations and infection prevalence in host populations for amphibian parasite *Ribeiroia ondatrae* (Huver et al. 2015) and *A. astaci* (Strand et al. 2014), *Ceratomyxa shasta* induced mortality in salmonids (Hallett et al. 2012) and frequency of gastrointestinal illness among swimmers due to *Enterococcus* spp. (Wade et al. 2010). However, further experimental and empirical data is needed to further elucidate a quantitative or semi-quantitative relationship between disease agent DNA concentrations in water and disease prevalence and risk in host populations. The relation between the occurrence of one or several parasites in an aquatic system and that system's properties and adequacy for host populations therefore warrants further inspection. Detection of parasite communities and their hosts in water using eDNA-based techniques can facilitate further research in this area.

Inhibition of water samples

Signs of inhibition were rare in water samples, which could be either due to low amounts of inhibitors in the water or through efficient inhibitor removal during DNA extraction. Nevertheless, inhibited samples showed lower detection rates and DNA concentrations of *S. parasitica* and lower DNA concentrations for *T. bryosalmonae*. Most sites where inhibition was observed were smaller rivers, i.e. rivers of Strahler order 4 or lower (n = 7 / 13), which might experience a larger input of inhibitory compounds such as humic acids from leaf litter (Lance & Guan 2020), proportionally to their size than big rivers or lakes. However, the low number of inhibited sites makes conclusive interpretation of this pattern challenging. Nevertheless, these findings indicate even though inhibition was not frequent in water samples, it should not be disregarded as a factor that could influence pathogen eDNA survey results.

Conclusions

We conducted a first country-wide survey of Switzerland of four different pathogens using eDNA-based techniques. We could confirm the widespread distribution of *A. astaci* and *T. bryosalmonae* observed in previous surveys (Wahli et al. 2008, Jean-Richard 2013). Rare detection of *B. dendrobatidis* was likely due to sampling site selection which did not target amphibian habitats. For future surveys of *B. dendrobatidis* we advise selection of suitable or known amphibian habitats and modifying the sampling scheme to more, but lower volume, samples to mitigate problems with filter clogging. This further implies that, while surveying multiple pathogens in water has been shown to be feasible in this study, the range of pathogens might still be limited by host properties, such as habitat range. *S. parasitica* was present in most of the surveyed sites, confirming its ubiquitous nature. A major outstanding problem in using eDNA measurements to ultimately inform on disease risk is the largely unknown dynamic between disease agent DNA concentrations in water, often near detection limits, and disease prevalence in host populations. Here, more accurate quantification methods (e. g. digital droplet PCR, Whale et al. 2012), experimental data and field method comparisons, could prove useful for maximising gain of information from eDNA studies and show the degree of reliability of eDNA quantitative data. Monitoring campaigns of aquatic diseases using conventional methods are cost- and time-intensive and different methods are applied for different species. However, such studies combined with eDNA-based monitoring techniques are the key to interpreting the relatively easily acquired pathogen eDNA detections and quantifications in water. Nevertheless, applicability of the eDNA method with only minor changes for a wide range of species raises the opportunity of more frequent, comprehensive disease monitoring schemes and investigation of not only host-parasite coexistence, but also parasite co-occurrences. We conclude that careful compilation of environmental data and conventional disease monitoring methods, in addition to data gleaned from eDNA, would increase our understanding on factors influencing host-parasite and parasite coexistence and therefore help in devising appropriate and effective disease management plans.

Acknowledgements We thank Marta Reyes, Jana Jucker, Raffael Stegmayer, Pravin Ganesanandamoorthy, Sarah Bratschi and Tamara Schlegel for their assistance during sampling. We further thank Anna Weston for assistance in the lab. Finally, we thank Prof.

Jukka Jokela from ETH and the Swiss Federal Government for the Environment (FOEN) for their funding.

Literature cited

Adrian-Kalchhauser I, Burkhardt-Holm P (2016) An eDNA assay to monitor a globally invasive fish species from flowing freshwater. PLoS One 11:1–22

Alderman DJ, Polglase JL, Frayling M (1987) *Aphanomyces astaci* pathogenicity under laboratory and field conditions. J Fish Dis 10:385–393

Anglès d'Auriac MB, Strand DA, Mjelde M, Demars BOL, Thaulow J (2019) Detection of an invasive aquatic plant in natural water bodies using environmental DNA. PLoS One 14:1–15

Audemard C, Reece KS, Burreson EM (2004) Real-time PCR for detection and quantification of the protistan parasite *Perkinsus marinus* in environmental waters. Appl Environ Microbiol 70:6611–6618

Aw TG, Rose JB (2012) Detection of pathogens in water: from phylochips to qPCR to pyrosequencing. Curr Opin Biotechnol 23:422–430

BAFU (2004) Methoden zur Untersuchung und Beurteilung der Fliessgewässer - Fische Stufe F (flächendeckend). Bern.

BAFU (2019) Methoden zur Untersuchung und Beurteilung der Fliessgewässer - Makrozoobenthos - Stufe F (flächendeckend). Bern

BAFU (1998) Methoden zur Untersuchung und Beurteilung der Fliessgewässer - Ökomorphologie Stufe F (flächendeckend). Bern.

Bailey C, Schmidt-Posthaus H, Segner H, Wahli T, Strepparava N (2018) Are brown trout *Salmo trutta fario* and rainbow trout *Oncorhynchus mykiss* two of a kind? A comparative study of salmonids to temperature-influenced *Tetracapsuloides bryosalmonae* infection. J Fish Dis 41:191–198

Barnes MA, Brown AD, Daum MN, de la Garza KA, Driskill J, Garrett K, Goldstein MS, Luk A, Maguire JI, Moke R, Ostermaier EM, Sanders YM, Sandhu T, Stith A, Suresh V V. (2020) Detection of the amphibian pathogens chytrid fungus (*Batrachochytrium*

dendrobatidis) and ranavirus in West Texas, USA, using environmental DNA. J Wildl Dis 56:702–706

van den Berg AH, McLaggan D, Diéguez-Uribeondo J, van West P (2013) The impact of the water moulds *Saprolegnia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. Fungal Biol Rev 27:33–42

Biggs J, Ewald N, Valentini A, Gaboriaud C, Dejean T, Griffiths RA, Foster J, Wilkinson JW, Arnell A, Brotherton P, Williams P, Dunn F (2015) Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). Biol Conserv 183:19–28

Bohman P, Nordwall F, Edsman L (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. Bull Fr la Pêche la Piscic 380–381:1291–1302

Bonadonna L, Briancesco R, La Rosa G (2019) Innovative analytical methods for monitoring microbiological and virological water quality. Microchem J 150:1–8

Carraro L, Hartikainen H, Jokela J, Bertuzzo E, Rinaldo A (2018) Estimating species distribution and abundance in river networks using environmental DNA. Proc Natl Acad Sci USA 115:11724–11729

Chestnut T, Anderson C, Popa R, Blaustein AR, Voytek M, Olson DH, Kirshtein J (2014) Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS One 9

Chucholl C (2017) Niche-based species distribution models and conservation planning for endangered freshwater crayfish in south-western Germany. Aquat Conserv Mar Freshw Ecosyst 27:698–705

Cunningham AA, Daszak P, Wood JLN, Cunningham AA (2017) One Health , emerging infectious diseases and wildlife : two decades of progress ? Philos Trans R Soc B Biol Sci 372:20160167

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science (80-) 287:443–449

Daszak P, Strieby A, Cunningham AA, Longcore JE, Brown CC, Porter D (2004) Experimental evidence that the bullfrog (*Rana catesbeiana*) is a potential carrier of

chytridiomycosis, an emerging fungal disease of amphibians. Herpetol J 14:201–207

Doi H, Katano I, Sakata Y, Souma R, Kosuge T, Nagano M, Ikeda K, Yano K, Tojo K (2017) Detection of an endangered aquatic heteropteran using environmental DNA in a wetland ecosystem. R Soc Open Sci 4

Dorazio RM, Erickson RA (2018) Ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380

Edgerton BF, Evans LH, Stephens FJ, Overstreet RM (2002) Review article: synopsis of freshwater crayfish diseases and commensal organisms. Aquac 206:57–135

Fontes I, Hartikainen H, Holland JW, Secombes CJ, Okamura B (2017) *Tetracapsuloides bryosalmonae* abundance in river water. Dis Aquat Org 124:145–157

Gelfand AE, Ghosh SK (1998) Model choice: a minimum posterior predictive loss approach. Biometrika 85:1–11

Hallett SL, Bartholomew JL (2006) Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Dis Aquat Org 71:109–118

Hallett SL, Ray RA, Hurst CN, Holt RA, Buckles GR, Atkinson SD, Bartholomew JL (2012) Density of the waterborne parasite *Ceratomyxa shasta* and its biological effects on salmon. Appl Environ Microbiol 78:3724–3731

Harper KJ, Anucha NP, Turnbull JF, Bean CW, Leaver MJ (2018) Searching for a signal: environmental DNA (eDNA) for the detection of invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). Manag Biol Invasions 9:137–148

Holdich DM (2003) Crayfish in Europe - an overview of taxonomy, legislation, distribution, and crayfish plague outbreaks. Proceedings of a conference held on 7th November, 2002. In: *Management & Conservation of Crayfish*. Holdich DM, Sibley PJ (eds) Bristol, p 15–34

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl Manag Aquat Ecosyst 2009:394–395

Howe GE, Stehly GR (1998) Experimental infection of rainbow trout with *Saprolegnia*

parasitica. J Aquat Anim Health 10:397–404

Hutchins PR, Sepulveda AJ, Martin RM, Hopper LR (2018) A probe-based quantitative PCR assay for detecting *Tetracapsuloides bryosalmonae* in fish tissue and environmental DNA water samples. Conserv Genet Resour 10:317–319

Huver JR, Koprivnikar J, Johnson PTJ, Whyard S (2015) Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. Ecol Appl 25:991–1002

Hyman OJ, Collins JP (2012) Evaluation of a filtration-based method for detecting *Batrachochytrium dendrobatidis* in natural bodies of water. Dis Aquat Org 97:185–195

Jean-Richard P (2013) Krebspesterhebung in der Schweiz Kampagne 2012. BAFU, Bern:13

Kamoroff C, Goldberg CS (2017) Using environmental DNA for early detection of amphibian chytrid fungus *Batrachochytrium dendrobatidis* prior to a ranid die-off. Dis Aquat Org 127:75–79

Kilpatrick AM, Briggs CJ, Daszak P (2010) The ecology and impact of chytridiomycosis: an emerging disease of amphibians. Trends Ecol Evol 25:109–118

Kirshtein JD, Anderson CW, Wood JS, Longcore JE, Voytek MA (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Dis Aquat Org 77:11–15

Kitancharoen N, Yuasa K, Hatai K (1996) Effects of pH and temperature on growth of *Saprolegnia diclina* and *S. parasitica* isolated from various sources. Mycoscience 37:385–390

Kozubíková E, Filipová L, Kozák P, Ďuriŝ Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B, Petrusek A (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech republic. Conserv Biol 23:1204–1213

Kozubíková E, Viljamaa-Dirks S, Heinikainen S, Petrusek A (2011) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. J Invertebr Pathol 108:214–216

Krieg R, King A, Sieber N, Vorburger C, Hartikainen H, Zenker A (2019) Nachweis von Wassertierkrankheiten mit Hilfe der sogenannten Umwelt-DNA (eDNA) Methode aus Wasserproben.

Kunz M, Schindler Wildhaber Y, Dietzel A (2016) Zustand der Schweizer Fliessgewässer - Ergebnisse der Nationalen Beobachting Oberflächengewässerqualität (NAWA) 2011-2014. BAFU, Bern.

Lance RF, Guan X (2020) Variation in inhibitor effects on qPCR assays and implications for eDNA surveys. Can J Fish Aquat Sci 77:23–33

Lips KR, Brem F, Brenes R, Reeve JD, Alford RA, Voyles J, Carey C, Livo L, Pessier AP, Collins JP (2006) Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proc Natl Acad Sci USA 103:3165–3170

Longcore JE, Pessier AP, Nichols DK (1999) *Batrachochytrium dendrobatidi*s gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219–227

Mächler E, Deiner K, Steinmann P, Altermatt F (2014) Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshw Sci 33:1174–1183

Marcogliese DJ (2008) The impact of climate change on the parasites and infectious diseases of aquatic animals.pdf. Rev Sci Tech Paris OIE (Office Int des Epizoot 27:467–484

Mauvisseau Q, Burian A, Gibson C, Brys R, Ramsey A, Sweet M (2019) Influence of accuracy, repeatability and detection probability in the reliability of species-specific eDNA based approaches. Sci Rep 9:1–10

Mitchell SE, Rogers ES, Little TJ, Read AF (2005) Host-parasite and genotype-by-environment interactions: temperature modifies potential for selection by a sterilizing pathogen. Evolution (N Y) 59:70–80

Mosher BA, Huyvaert KP, Bailey LL (2018) Beyond the swab: ecosystem sampling to understand the persistence of an amphibian pathogen. Oecologia 188:319–330

Neish GA (1977) Observations on saprolegniasis of adult sockeye salmon, *Oncorhynchus nerka* (Walbaum). J Fish Biol 10:513–522

Nyhlén L, Unestam T (1980) Wound reactions and *Aphanomyces astaci* growth in crayfish

cuticle. J Invertebr Pathol 36:187–197

Okamura B, Hartikainen H, Schmidt-Posthaus H, Wahli T (2011) Life cycle complexity, environmental change and the emerging status of salmonid proliferative kidney disease. Freshw Biol 56:735–753

Paul C, Belbahri L (2012) Clonalité de *Saprolegnia parasitica*, le parasite des poissons du Doubs.

Pawlowski J, Apothéloz-Perret-Gentil L, Altermatt F (2020) Environmental (e)DNA: what's behind the term? Clarifying the terminology and recommendations for its future use in biomonitoring. Mol Ecol:mec.15643

Peeler EJ, Oidtmann BC, Midtlyng PJ, Miossec L, Gozlan RE (2011) Non-native aquatic animals introductions have driven disease emergence in Europe. Biol Invasions 13:1291–1303

Pickering A, Willoughby L (1982) *Saprolegnia* infections of salmonid fish. In: *Fiftieth Annual Report*. Freshwater Biological Association, Ambleside, UK, p 38–49

Quiniou SMA, Bigler S, Clem LW, Bly JE (1998) Effects of water temperature on mucous cell distribution in channel catfish epidermis: A factor in winter saprolegniasis. Fish Shellfish Immunol 8:1–11

R Core Team (2019) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.r-project.org/

Ravasi D, De Respinis S, Wahli T (2018) Multilocus sequence typing reveals clonality in *Saprolegnia parasitica* outbreaks. J Fish Dis 41:1653–1665

Richey CA, Kenelty K V, Hopkins KVS, Stevens BN, Mart B, Barnum SM, Hallett SL, Atkinson SD, Bartholomew JL, Soto E (2018) Distribution and prevalence of Myxobolus cerebralis in post fire areas of Plumas National Forest : utility of environmental DNA sampling. J Aquat Anim Health 30:130–143

Rocchi S, Tisserant M, Valot B, Laboissière A, Frossard V, Reboux G (2017) Quantification of *Saprolegnia parasitica* in river water using real-time quantitative PCR: from massive fish mortality to tap drinking water. Int J Environ Health Res 27:1–10

Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66:2541–2547

Rusch JC, Hansen H, Strand DA, Markussen T, Hytterød S, Vrålstad T (2018) Catching the fish with the worm: A case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Parasites and Vectors 11:1–12

Schmidt BR, Kéry M, Ursenbacher S, Hyman OJ, Collins JP (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. Methods Ecol Evol 4:646–653

Strand D, Rusch J, Tarpai A, Johnsen SI, Vrålstad T (2019a) The surveillance programme for *Aphanomyces astac*i in Norway 2018.

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Dis Aquat Org 95:9–17

Strand DA, Johnsen SI, Rusch JC, Agersnap S, Larsen WB, Knudsen SW, Møller PR, Vrålstad T (2019b) Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. J Appl Ecol 56:1661–1673

Strand DA, Jussila J, Johnsen SI, Viljamaa-Dirks S, Edsman L, Wiik-Nielsen J, Viljugrein H, Engdahl F, Vrålstad T (2014) Detection of crayfish plague spores in large freshwater systems. J Appl Ecol 51:544–553

Stucki P, Zaugg B (2011) Aktionsplan Flusskrebse Schweiz. Artenförderung von Edelkrebs, Dohlenkrebs und Steinkrebs. Bern.

Thomsen PF, Kielgast J, Iversen LL, Møller PR, Rasmussen M, Willerslev E (2012) Detection of a diverse marine fish fauna using environmental DNA from seawater samples. PLoS One 7:e41732

Thomsen PF, Willerslev E (2015) Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. Biol Conserv 183:4–18

Tobler U, Borgula A, Schmidt BR (2012) Populations of a susceptible amphibian species can grow despite the presence of a pathogenic chytrid fungus. PLoS One 7:1–8

Tops S, Hartikainen HL, Okamura B (2009) The effects of infection by *Tetracapsuloides bryosalmonae* (Myxozoa) and temperature on *Fredericella sultana* (Bryozoa). Int J Parasitol 39:1003–1010

Toranzos GA, Alvarez AJ, Dvorsky EA (1993) Application of the polymerase chain reaction technique to the detection of pathogens in water. Water Sci Technol 27:207–210

Tukey J (1977) Exploratory Data Analysis. Addison-Wesley, Reading MA.

Viljamaa-Dirks S, Heinikainen S (2019) A tentative new species *Aphanomyces fennicus* sp. nov. interferes with molecular diagnostic methods for crayfish plague. J Fish Dis 42:413–422

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:146–155

Wade TJ, Sams E, Brenner KP, Haugland R, Chern E, Beach M, Wymer L, Rankin CC, Love D, Li Q, Noble R, Dufour AP (2010) Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. Environ Heal A Glob Access Sci Source 9:1–14

Wahli T, Bernet D, Segner H, Schmidt-Posthaus H (2008) Role of altitude and water temperature as regulating factors for the geographical distribution of *Tetracapsuloides bryosalmonae* infected fishes in Switzerland. J Fish Biol 73:2184–2197

Wahli T, Bernet D, Steiner PA, Schmidt-Posthaus H (2007) Geographic distribution of *Tetracapsuloides bryosalmonae* infected fish in Swiss rivers: An update. Aquat Sci 69:3–10

Wahli T, Knuesel R, Bernet D, Segner H, Pugovkin D, Burkhardt-Holm P, Escher M, Schmidt-Posthaus H (2002) Proliferative kidney disease in Switzerland: Current state of knowledge. J Fish Dis 25:491–500

Walker SF, Salas MB, Jenkins D, Garner TWJ, Cunningham AA, Hyatt AD, Bosch J, Fisher MC (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a

temperate climate. Dis Aquat Org 77:105–112

Watanabe S (2010) Asymptotic equivalence of Bayes cross validation and widely applicable information criterion in singular learning theory. J Mach Learn Res 11:3571–3594

van West P (2006) *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 20:99–104

Whale AS, Huggett JF, Cowen S, Speirs V, Shaw J, Ellison S, Foy CA, Scott DJ (2012) Comparison of microfluidic digital PCR and conventional quantitative PCR for measuring copy number variation. Nucleic Acids Res 40

Wittwer C, Stoll S, Strand D, Vrålstad T, Nowak C, Thines M (2018) EDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia 807:87–97

Wittwer C, Stoll S, Thines M, Nowak C (2019) EDNA-based crayfish plague detection as practical tool for biomonitoring and risk assessment of *A. astaci*-positive crayfish populations. Biol Invasions 21:1075–1088

Supplementary Material Chapter 3

Text S1. Sequence of double-stranded Gblocks fragment used to create the standard curve for quantitative PCR. Forward and reverse primers are marked in **bold**, probes in **bold** and *italic* for *A. astaci*, *B. dendrobatidis*, *S. parasitica* and *T. bryosalmonae*.

5'-
AAGGCTTGTGCTGGGATGTTC***TTCGGGACGACCC***GGC**TAGCAGAAGGTTTCGCAAGAAG**TTTTT**CCTTGATATAATA**
CAGTGTGCCATATGTCA***CGAGTCGAACAAAAT***TTATTTATTTTTTCGACAAATTAATTGGAAATTGAATAATTTAATTG
AAAAAAATTGAAAATAAATATTAAAAACAACT**TTTGACAACGGATCTCTTGGCT**TTTTT**AGAGCAAATCGCGGTAGTTT**
TGCTTGTACTTCGGTACGAGTGGACACATATTGCTTTTTGTGATTTCT***GCGAGTCTGTTGTCAAAGTACAAGGCA***CGTA
AGGAGAGT**TGGTATGCTGGTGCATTTCT**TTTTT**GGTTGTTTAGTTTGGGCTCACC**ATATGTATGTTGTTGGTTTAGACA
CTGATA***CAAGATCTTATTTTATGGCTGCCAC***TATGA**CAATAGCTGTCCCTACAGGGA** -3'

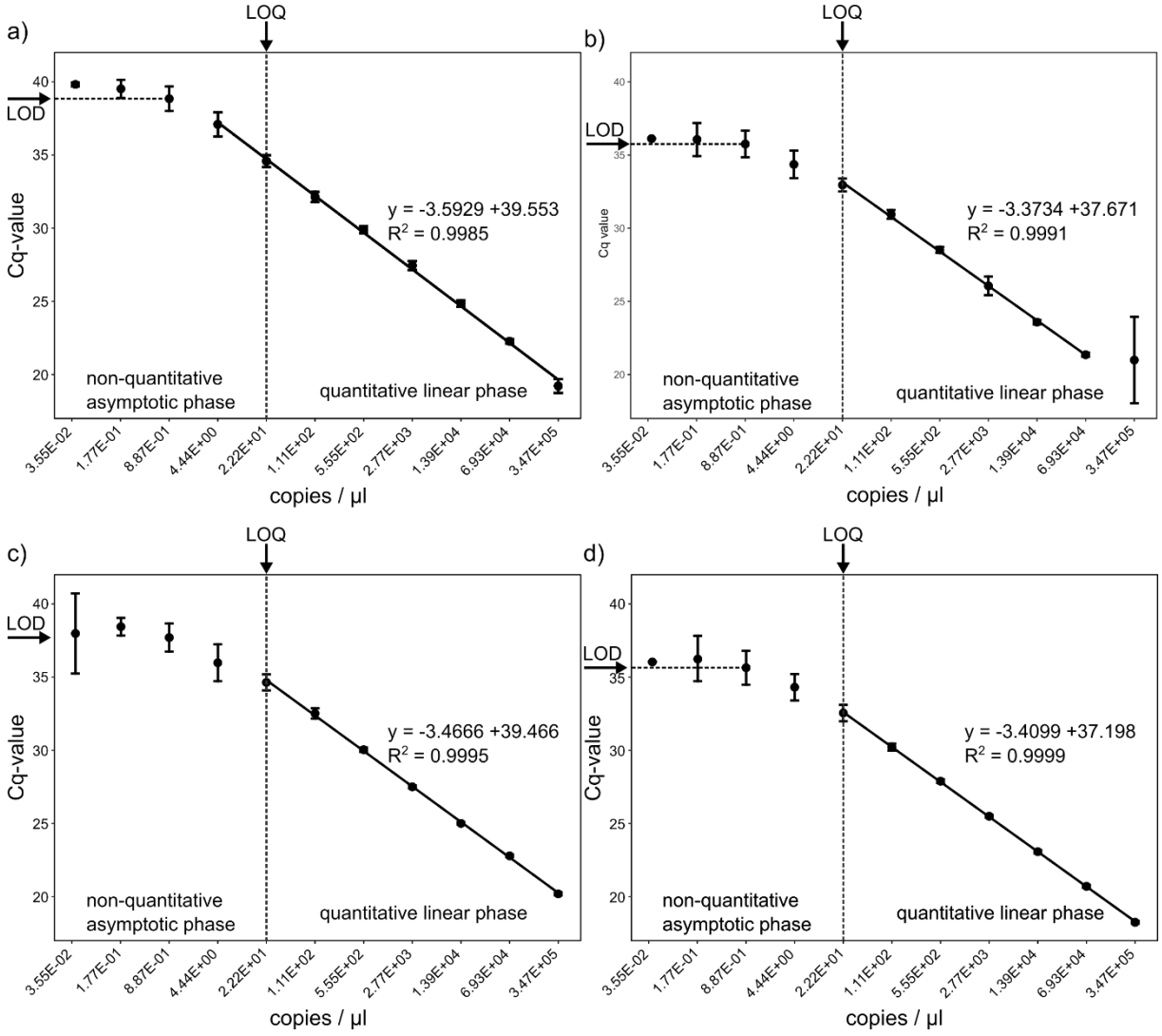


Figure S1. Standard curve used for defining the limit of detection (LOD) and limit of quantification (LOQ) for a) *A. astaci*, b) *S. parasitica*, c) *B. dendrobatidis* and d) *T. bryosalmonae*. The strongest dilution of *S. parasitica* was omitted from the curve due to high variability in Cq-values.

Table S1. List of water sampling sites including name of waterbody, canton, coordinates (CH1903 LV03), waterbody type, elevation, catchment, river Strahler order, lake size = lake surface area in km^2, slope categories, ecosystem integrity criteria (ecomorphology, IBCH = invertebrates, fish community). Further, for each pathogen, mean DNA concentrations in water in copies µl^{-1}, positive / negative (1 / 0) detection below and / or above limit of detection (LOD) are listed per site.

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
Aabach	LU	659827	233069	river	450	Aare	4	NA	flach	3	NA	NA	0.061	1	1	NA	0	0	33.364	1	1	1.291	1	1
Aabach	ZH	695479	244545	river	460	Rhein	5	NA	mittelsteil	1	2	3	0.129	1	0	NA	0	0	25.538	1	1	NA	0	0
Aabach	SG	714846	231225	river	410	Limmat	6	NA	mittelsteil	NA	NA	NA	0.000	1	0	NA	0	0	133.393	1	1	6.925	1	1
Aabach	AG	654895	251775	river	387	Aare	4	NA	mittelsteil	3	3	NA	3.747	1	1	NA	0	0	381.325	1	1	28.432	1	1
Aare-Hagneck-Kanal	BE	582133	210628	river	439	Aare	9	NA	flach	2	NA	NA	0.250	1	1	NA	0	0	323.262	1	1	10.232	1	1
Aare	BE	594528	221242	river	426	Aare	3	NA	flach	2	NA	NA	0.058	1	0	NA	0	0	10.306	1	1	NA	0	0
Aare	SO	608900	229570	river	426	Aare	9	NA	flach	2	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Aare	BE	657081	174886	river	596	Aare	6	NA	flach	2	3	2	0.163	1	1	NA	0	0	30.418	1	1	NA	0	0
Aare	SO	608600	229000	river	426	Aare	9	NA	flach	2	NA	NA	0.706	1	1	NA	0	0	11.216	1	1	NA	0	0
Aare	AG	646659	250311	river	384	Aare	9	NA	mittelsteil	3	NA	NA	2.090	1	1	NA	0	0	126.134	1	1	18.307	1	1
Aare	BE	618887	231528	river	418	Aare	9	NA	NA	2	NA	NA	0.143	1	1	NA	0	0	NA	0	0	NA	0	0
Aare	BE	617093	231574	river	418	Aare	9	NA	NA	2	NA	NA	8.976	1	1	NA	0	0	255.459	1	1	NA	0	0
Ägerisee	ZG	687249	221295	lake	724	Reuss	NA	7.2	NA	NA	NA	NA	12.316	1	0	NA	0	0	NA	0	0	NA	0	0
Albula	GR	755266	174496	river	676	Alpenrhein	6	NA	mittelsteil	4	NA	NA	NA	0	0	NA	0	0	0.000	1	1	NA	0	0
Allaine	JU	577802	252909	river	457	Doubs	3	NA	mittelsteil	3	NA	NA	3.020	1	1	NA	0	0	30.259	1	1	10.268	1	1
Allaine	JU	568089	260720	river	367	Doubs	5	NA	flach	1	3	3	0.087	1	0	NA	0	0	146.588	1	1	4.134	1	1
Allondon	GE	490024	120712	river	417	Rhône	3	NA	mittelsteil	4	NA	NA	8.810	1	1	NA	0	0	96.979	1	1	32.061	1	1
Allondon	GE	488587	118003	river	381	Rhône	3	NA	mittelsteil	NA	NA	NA	0.285	1	1	NA	0	0	16.765	1	1	7.921	1	1
Allondon	GE	489615	115922	river	356	Rhône	3	NA	mittelsteil	3	NA	NA	NA	NA	NA	NA	0	0	377.425	1	1	8.789	1	1
Alpenrhein	GR	760592	200693	river	526	Alpenrhein	8	NA	flach	3	NA	NA	0.139	1	0	NA	0	0	155.153	1	1	NA	0	0
Areuse	NE	553724	200788	river	455	Aare	4	NA	flach	2	3	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Aubonne	VD	520742	147728	river	394	Rhône	4	NA	mittelsteil	4	3	3	NA	NA	NA	NA	0	0	453.092	1	1	NA	0	0
Bach bei San Carpoforo	TI	723120	120249	river	233	Ticino	1	NA	flach	2	NA	NA	1.637	1	0	NA	0	0	4784.099	1	1	NA	0	0
Bach bei Scereda	TI	719086	83146	river	288	Ticino	5	NA	mittelsteil	2	NA	NA	1.560	1	0	NA	0	0	934.510	1	1	NA	0	0
Baggerseeli	BE	634507	171173	lake	564	Aare	NA	0.011	NA	NA	NA	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
Baldeggersee	LU	662537	229405	lake	463	Aare	NA	5.3	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	NA	NA	NA	0	0
Berentalbach	ZH	699234	258434	river	476	Rhein	2	NA	mittelsteil	2	NA	NA	8.282	1	1	NA	0	0	419.640	1	1	3.376	1	1
Bief du Lavoir d. l. p. Grave	GE	492581	115183	river	413	Rhône	1	NA	mittelsteil	4	NA	NA	NA	0	0	NA	0	0	7.874	1	1	NA	0	0
Bief du Lavoir d. l. p. Grave	GE	492836	115378	river	399	Rhône	1	NA	steil	3	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Bielersee	BE	577001	214760	lake	429	Aare	NA	39.8	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Bielersee	BE	581598	218139	lake	429	Aare	NA	39.8	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Bienzerliweiher	ZH	672774	252778	lake	381	Limmat	NA	0.0111	NA	NA	NA	NA	3.834	1	0	NA	0	0	64.569	1	1	NA	0	0

Table S1. (cont.)

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
Birs	BL	612790	256385	river	297	Rhein		6	NA	flach		2	NA	NA	28.196	1	1	NA	0	0	94.528	1	1	1.448	1	1
Birs	BL	613685	263163	river	267	Rhein		6	NA	flach		2	2	NA	107.844	1	1	NA	0	0	29.818	1	1	0.522	1	0
Birsig	BL	609780	263463	river	289	Rhein		4	NA	flach		1	NA	NA	0.003	1	0	NA	0	0	4.401	1	0	NA	0	0
Birsig	BL	608481	261766	river	299	Rhein		4	NA	mittelsteil		3	NA	NA	0.105	1	1	NA	0	0	58.411	1	1	19.017	1	1
Birsig	BL	609092	262535	river	294	Rhein		4	NA	flach		1	NA	NA	165.768	1	1	NA	0	0	19.644	1	1	17.619	1	1
Bodensee	TG	746476	270368	lake	385	Rhein	NA	541.2	NA	NA		NA	NA		2.049	1	0	NA	0	0	146.334	1	1	NA	0	0
Bodensee	SG	755558	260697	lake	393	Rhein	NA	541.2	NA	NA		NA	NA	NA		0	0	NA	0	0	55.557	1	1	NA	0	0
Bodensee	TG	716107	281003	lake	390	Rhein	NA	541.2	NA	NA		NA	NA		3.646	1	0	NA	0	0	1853.663	1	1	NA	0	0
Bodensee	SG	750967	263535	lake	358	Rhein	NA	541.2	NA	NA		NA	NA	NA		0	0	NA	0	0	NA	0	0	NA	0	0
Boiron de Nyon	VD	502874	136372	river	455	Rhône		3	NA	mittelsteil		4	NA	NA	28.540	1	1	NA	0	0	30.302	1	1	NA	0	0
Bolla Rossa	TI	710711	113829	river	204	Ticino		4	NA	flach		2	NA	NA	NA	0	0	NA	0	0	1104.483	1	1	NA	0	0
Brenno	TI	718140	137726	river	356	Ticino		5	NA	mittelsteil		4	NA	NA	0.439	1	0	NA	0	0	1430.623	1	1	NA	0	0
Brienzersee	BE	634150	171374	lake	564	Aare	NA	29.8	NA	NA		NA	NA		0.754	1	1	NA	0	0	NA	0	0	NA	0	0
Broye	VD	566155	191903	river	431	Aare		6	NA	flach		2	2	NA	0.583	1	0	NA	0	0	0.391	1	1	NA	0	0
Broye	VD	554520	155034	river	652	Aare		5	NA	mittelsteil		4	NA	NA	NA	NA	NA	NA	0	0	5.220	1	1	0.872	1	0
Broye	VD	560370	182567	river	451	Aare		6	NA	flach		2	NA	NA	0.009	1	0	NA	0	0	NA	0	0	NA	0	0
Broye	VD	550411	167900	river	513	Aare		6	NA	mittelsteil		1	NA	NA	NA	NA	NA	1.130685155	1	0	236.560	1	1	0.029	1	1
Bünz	AG	656343	251312	river	374	Aare		5	NA	mittelsteil		3	2	1	2.563	1	1	NA	0	0	62.981	1	1	104.001	1	1
Buttisholz	LU	648658	215899	lake	596	Aare	NA	0.24	NA	NA		NA	NA	NA		0	0	NA	0	0	NA	0	0	NA	0	0
Buuserbach	BL	629702	263860	river	350	Rhein		3	NA	mittelsteil		3	NA	NA	0.232	1	1	NA	0	0	1494.076	1	1	NA	0	0
Canale sponda sinistra	TI	715344	113442	river	208	Ticino		4	NA	flach		2	NA	NA	0.924	1	0	7.623332733	1	1	5563.634	1	1	NA	0	0
Carrouge	VD	549911	162714	river	665	Aare		4	NA	mittelsteil		4	NA	NA	0.006	1	0	4.037983542	1	0	NA	NA	NA	NA	0	0
Chäppelibach	SZ	688128	210874	river	450	Reuss		2	NA	mittelsteil		1	NA	NA	0.415	1	1	NA	0	0	2687.784	1	1	NA	0	0
Chatzentobel-Weiher	ZH	700385	233027	lake	444	Limmat	NA	0.0052	NA	NA		NA	NA		1.620	1	0	NA	0	0	NA	0	0	NA	0	0
Chli Linthli	GL	723422	219371	river	430	Limmat		2	NA	flach		2	NA	NA	0.005	1	0	NA	0	0	1.064	1	1	NA	0	0
Chuesenbach	ZH	687298	242380	river	529	Limmat		1	NA	steil		3	NA	NA	2.996	1	0	NA	0	0	1080.304	1	1	NA	0	0
Dättwilerweiher	AG	664072	257253	lake	438	Limmat	NA	0.011	NA	NA		NA	NA	NA		0	0	NA	0	0	11.534	1	1	NA	0	0
Dorfbach	ZH	691153	278325	river	410	Rhein		1	NA	NA		1	NA	NA	3.717	1	0	NA	0	0	45.796	1	1	NA	0	0
Doubs	JU	572586	244466	river	419	Doubs		5	NA	flach		3	4	NA	2.580	1	1	NA	0	0	47.150	1	1	NA	0	0
Dünnern	SO	629548	241889	river	425	Aare		5	NA	flach		2	2	2	NA	0	0	NA	0	0	1.833	1	0	NA	0	0
Eaux-Chaudes	GE	489512	116326	river	360	Rhône		2	NA	mittelsteil	NA		NA	NA	1.368	1	0	NA	0	0	0.047	1	0	NA	0	0
Eaux-Chaudes	GE	489627	115938	river	357	Rhône		3	NA	mittelsteil		4	NA	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
Eaux-Froides	GE	489598	115884	river	360	Rhône		3	NA	mittelsteil	NA		NA	NA	NA	NA	NA	NA	0	0	0.094	1	1	NA	0	0
Egelsee	BE	601991	199308	lake	549	Aare	NA	0.015	NA	NA		NA	NA	NA		NA	NA	NA	0	0	NA	0	0	NA	0	0
Emme	SO	609479	225479	river	445	Aare		7	NA	flach		2	3	NA	0.231	1	1	NA	0	0	1.984	1	0	NA	0	0
Ergolz	BL	629884	256865	river	383	Rhein		4	NA	mittelsteil		3	NA	NA	0.088	1	0	NA	0	0	586.953	1	1	173.136	1	1
Ergolz	BL	625220	257967	river	333	Rhein		5	NA	mittelsteil		3	2	2	0.082	1	0	NA	0	0	959.293	1	1	45.891	1	1
Erlibach	LU	677973	219030	river	414	Reuss		3	NA	flach	NA		NA	NA	NA	0	0	NA	0	0	3.242	1	1	NA	0	0
Erveratte	JU	577822	252739	river	457	Doubs		3	NA	mittelsteil		2	NA	NA	1.558	1	1	NA	0	0	93.776	1	1	14.700	1	1
Etang de Bonfol	JU	578958	257732	river	437	Doubs		2	NA	mittelsteil		3	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0

Table S1. (cont.)

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish		DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
Fallopia	TI	721276	77151	river	244	Adda		4	NA	NA		2	NA	NA	3.753	1	1	NA	0	0	1226.428	1	1	NA	0	0
Fencherengiessen	BE	589987	216057	lake	434	Aare	NA	0.025	NA	NA		NA	NA		1.400	1	1	NA	0	0	NA	0	0	NA	0	0
Fischbach	ZH	677517	259990	river	410	Rhein		1	NA	flach		1	NA	NA	0.003	1	0	NA	0	0	39.200	1	1	5.518	1	1
Frenke	BL	623169	258926	river	316	Rhein		5	NA	mittelsteil		2	NA	NA	0.014	1	0	NA	0	0	2307.630	1	1	NA	0	0
Furtbach	ZH	677498	255431	river	431	Limmat		2	NA	mittelsteil		3	NA	NA	2.369	1	0	2.210912721	1	1	618.702	1	1	0.278	1	1
Furtbach	ZH	679374	254642	river	440	Limmat		2	NA	flach		3	NA	NA	NA	0	0	NA	0	0	52.936	1	1	NA	0	0
Furtbach	AG	669576	254667	river	405	Limmat		4	NA	mittelsteil		2	1	2	6.140	1	1	NA	0	0	41.606	1	1	114.138	1	1
Füüla	VS	618476	128382	river	620	Rhône		2	NA	mittelsteil	NA		NA	NA	115.652	1	1	NA	0	0	78.945	1	1	NA	0	0
Genfersee	VD	541792	150503	lake	372	Rhône	NA	581.3	NA	NA		NA	NA		1.494	1	0	NA	0	0	1.635	1	1	NA	0	0
Genfersee	VD	557429	143553	lake	372	Rhône	NA	581.3	NA	NA		NA	NA		1.517	1	0	NA	0	0	8.991	1	1	NA	0	0
Genfersee	VD	508179	137589	lake	372	Rhône	NA	581.3	NA	NA		NA	NA		1.745	1	1	NA	0	0	119.889	1	1	NA	0	0
Giessenbach	ZH	705523	235593	river	490	Limmat		4	NA	mittelsteil		3	NA	NA	0.000	1	0	NA	0	0	23.507	1	1	23.013	1	1
Giessenparksee	SG	757525	208062	lake	502	Alpenrhein	NA	0.03	NA	NA		NA	NA	NA		0	0	NA	0	0	0.212	1	0	NA	0	0
Girhalde Weiher	ZH	694480	254150	lake	506	Rhein	NA	0.0013	NA	NA		NA	NA	NA		0	0	NA	0	0	1.984	1	0	NA	0	0
Glâne	FR	559728	169527	river	695	Aare		4	NA	flach		1	NA	NA	0.002	1	0	NA	0	0	571.290	1	1	11.723	1	1
Glâne	FR	556632	165551	river	739	Aare		3	NA	mittelsteil		3	NA	NA	NA	NA	NA	NA	0	0	654.430	1	1	NA	0	0
Glâne	FR	562639	173073	river	680	Aare		4	NA	flach		2	NA	NA	6.315	1	1	NA	0	0	NA	0	0	1.908	1	1
Glatt	SG	729558	255708	river	500	Rhein		6	NA	mittelsteil		4	2	2	21.614	1	1	0.001	1	0	186.736	1	1	30.177	1	1
Glatt	SG	729421	256077	river	498	Rhein		6	NA	mittelsteil		4	NA	NA	8.307	1	1	NA	0	0	122.043	1	1	6.528	1	1
Glatt	ZH	691263	248241	river	435	Rhein		6	NA	flach		3	2	NA	NA	0	0	NA	0	0	69.373	1	1	NA	0	0
Glatt	AR	737298	251360	river	670	Rhein		4	NA	mittelsteil		3	NA	NA	1.230	1	1	NA	0	0	754.353	1	1	NA	0	0
Goldach	SG	752257	259961	river	437	Rhein		5	NA	mittelsteil	NA		NA	NA	0.298	1	0	NA	0	0	198.394	1	1	NA	0	0
Greifensee	ZH	695115	241916	lake	480	Rhein	NA	8.6	NA	NA		NA	NA	NA		0	0	NA	0	0	0.074	1	1	NA	0	0
Greifensee	ZH	693770	243981	lake	435	Rhein	NA	8.6	NA	NA		NA	NA	NA		0	0	NA	0	0	26.703	1	1	NA	0	0
Greyerzesee	FR	574010	170479	lake	674	Aare	NA	9.6	NA	NA		NA	NA		0.805	1	0	NA	0	0	23.755	1	1	NA	0	0
Gross Aa	LU	657837	218961	river	510	Aare		4	NA	flach		3	NA	NA	NA	0	0	NA	0	0	0.177	1	1	9.679	1	1
Gründelisbach	SZ	689971	209628	river	449	Reuss		4	NA	mittelsteil		2	NA	NA	1.211	1	1	NA	0	0	463.358	1	1	NA	0	0
Gulantschi	VS	611482	128739	river	555	Rhône		2	NA	mittelsteil	NA		NA	NA	2.314	1	1	NA	0	0	355.599	1	1	NA	0	0
Hallwilersee	AG	657810	238051	lake	448	Aare	NA	10.3	NA	NA		NA	NA	NA		0	0	NA	0	0	NA	0	0	NA	0	0
Hinterrhein	GR	752803	165496	river	947	Alpenrhein		6	NA	mittelsteil		3	NA	NA	NA	0	0	NA	0	0	316.138	1	1	NA	0	0
Hinterrhein	GR	751355	181914	river	615	Alpenrhein		7	NA	flach		2	NA	NA	NA	0	0	NA	0	0	0.000	1	1	NA	0	0
Hongrin	VD	574408	139479	river	1403	Aare		5	NA	mittelsteil		4	NA	NA	1.423	1	1	NA	0	0	2.440	1	1	NA	0	0
Husemersee	ZH	695107	275231	lake	410	Rhein	NA	0.1	NA	NA		NA	NA		0.000	1	0	NA	0	0	NA	0	0	NA	0	0
Hüttenersee	ZH	693914	226715	lake	660	Limmat	NA	0.14	NA	NA		NA	NA		0.000	1	0	NA	0	0	3.888	1	1	NA	0	0
Inn	GR	831090	197862	river	1035	Inn		6	NA	NA		3	NA	NA	5.066	1	0	NA	0	0	50.922	1	1	96.618	1	0
Jona	ZH	711711	237782	river	550	Limmat		4	NA	mittelsteil		2	NA	NA	0.679	1	0	NA	0	0	429.260	1	1	1.619	1	1
Jonen	AG	671265	238823	river	383	Reuss		4	NA	mittelsteil		1	NA	NA	4.372	1	1	NA	0	0	17.106	1	1	0.172	1	1
Kander	BE	619088	163645	river	703	Aare		6	NA	mittelsteil		3	2	2	NA	NA	NA	0.021	1	0	4.808	1	1	NA	0	0
Katzensee	ZH	679534	253928	lake	439	Limmat	NA	0.305	NA	NA		NA	NA	NA		0	0	NA	0	0	9.637	1	1	NA	0	0
Kempt	ZH	695426	258339	river	450	Rhein		4	NA	mittelsteil		3	NA	NA	0.000	1	0	NA	0	0	191.831	1	1	16.849	1	1
Kleine Emme	LU	661952	211537	river	453	Reuss		7	NA	mittelsteil		2	3	NA	NA	0	0	NA	0	0	4.168	1	1	NA	0	0

Table S1. (cont.)

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
La Coeuvatte	JU	573527	259819	river	406	Doubs		4	NA	flach		3	NA	NA	0.242	1	0	NA		0	0	72.733	1	1	9.166	1	0
Lac de Brenet	VD	514254	169450	lake	1002	Aare	NA	9.56	NA	NA		NA	NA	6.172	1	1	NA		0	0	0.100	1	0	NA	0	0
Lac de Joux	VD	514775	169085	lake	1004	Aare	NA	9.56	NA	NA		NA	NA	NA	0	0	NA		0	0	NA	0	0	NA	0	0
Lac Ter	VD	512368	167609	lake	1017	Aare	NA	0.031	NA	NA		NA	NA	NA	0	0	NA		0	0	0.000	1	0	NA	0	0
Lago di Lugano	TI	717531	90161	lake	272	Ticino	NA	48.7	NA	NA		NA	NA	3.122	1	1	NA		0	0	536.506	1	1	NA	0	0
Lago di Lugano	TI	711190	91804	lake	262	Ticino	NA	48.7	NA	NA		NA	NA	9.461	1	1	NA		0	0	244.973	1	1	NA	0	0
Lago Maggiore	TI	698269	108162	lake	200	Ticino	NA	212.3	NA	NA		NA	NA	1.561	1	0	NA		0	0	116.244	1	1	NA	0	0
Landbach	ZH	677760	270955	river	376	Rhein		2	NA	flach		1	NA	NA	NA	0	0	NA		0	0	2.540	1	1	NA	0	0
Landquart	GR	766508	205009	river	580	Alpenrhein		6	NA	mittelsteil		3	3	NA	0.000	1	0		1.62E-05	1	0	33.824	1	1	NA	0	0
Lauerz	SZ	690249	209358	river	448	Reuss		5	NA	flach		2	NA	NA	0.264	1	1	NA		0	0	19.620	1	1	NA	0	0
Lauerzersee	SZ	687922	209560	lake	447	Reuss	NA	3.1	NA	NA		NA	NA	0.118	1	0	NA		0	0	15.428	1	1	NA	0	0
Laveggio	TI	719256	84526	river	261	Ticino		4	NA	mittelsteil		1	NA	NA	5.303	1	1	NA		0	0	286.696	1	1	33.609	1	1
Le Corbery	JU	578960	257726	river	437	Doubs		2	NA	mittelsteil		3	NA	NA	0.059	1	0	NA		0	0	NA	0	0	21.014	1	1
Limmat	ZH	672733	252780	river	382	Limmat		8	NA	flach		2	2	NA	4.009	1	0	NA		0	0	26.149	1	1	NA	0	0
Limmat	AG	666263	256432	river	363	Limmat		8	NA	NA		2	NA	NA	3.130	1	1	NA		0	0	14.404	1	1	NA	0	0
Linth-Kanal	GL	719562	225201	river	412	Limmat		7	NA	flach		2	NA	NA	0.033	1	0	NA		0	0	158.463	1	1	NA	0	0
Linth	GL	724826	219859	river	430	Limmat		6	NA	flach		2	NA	NA	0.133	1	1	NA		0	0	271.103	1	1	NA	0	0
Littibach	ZG	683697	228998	river	451	Reuss		4	NA	mittelsteil	NA		NA	NA	0.158	1	1	NA		0	0	928.315	1	1	NA	0	0
Lochgraben	SH	673337	283020	river	470	Rhein		2	NA	steil		4	NA	NA	0.000	1	0	NA		0	0	6.074	1	1	NA	0	0
Lorze	ZG	680543	225944	river	415	Reuss		6	NA	mittelsteil	NA		3	2	1.345	1	1	NA		0	0	886.680	1	1	8.644	1	1
Luthern	LU	640607	226452	river	488	Aare		5	NA	mittelsteil		2	NA	NA	0.187	1	0	NA		0	0	29.869	1	1	NA	0	0
Lütschine	BE	634489	170371	river	570	Aare		6	NA	mittelsteil		2	NA	NA	NA	NA	NA		0.002	1	0	8.924	1	1	NA	0	0
Lützel	BL	602010	252773	river	386	Rhein		4	NA	mittelsteil		2	NA	NA	2.290	1	1	NA		0	0	193.671	1	1	15.801	1	1
Lützel	BL	604183	251156	river	357	Rhein		4	NA	mittelsteil		2	NA	NA	7.450	1	1	NA		0	0	553.376	1	1	10.611	1	0
Maggia	TI	701234	115775	river	255	Ticino		7	NA	mittelsteil		4	3	NA	1.230	1	0	NA		0	0	56.927	1	1	NA	0	0
Maira	GR	762377	133859	river	793	Adda		5	NA	mittelsteil	NA		NA	NA	NA	0	0		6.529	1	1	78.411	1	1	NA	0	0
Mattenbach	ZH	700751	259312	river	483	Rhein		2	NA	mittelsteil		4	NA	NA	6.226	1	1	NA		0	0	187.383	1	1	7.643	1	1
Mattenbach	ZH	697731	261131	river	443	Rhein		3	NA	mittelsteil		2	NA	NA	3.006	1	0	NA		0	0	132.805	1	1	NA	0	0
Mauensee	LU	648143	224319	lake	504	Aare	NA	0.55	NA	NA		NA	NA	NA	0	0	NA		0	0	2633.608	1	1	NA	0	0
Meienriedsee	BE	592867	221154	lake	427	Aare	NA	0.063	NA	NA		NA	NA	0.658	1	0	NA		0	0	NA	0	0	NA	0	0
Mentue	VD	545386	180937	river	446	Aare		4	NA	mittelsteil		4	3	3	NA	0	0	NA		0	0	8.222	1	0	NA	0	0
Mentue	VD	544826	171892	river	560	Aare		4	NA	flach		4	NA	NA	NA	0	0	NA		0	0	NA	0	0	NA	0	0
Mettlenbach	ZH	697038	240610	river	460	Rhein		4	NA	mittelsteil		2	2	1	0.072	1	0	NA		0	0	0.000	1	1	NA	0	0
Moesa	GR	732612	123462	river	327	Ticino		5	NA	mittelsteil		4	3	NA	1.027	1	0	NA		0	0	425.492	1	1	NA	0	0
Mönchaltorfer Aa	ZH	696394	241463	river	410	Rhein		4	NA	flach	NA		2	1	0.192	1	0	NA		0	0	0.000	1	1	NA	0	0
Moossee	BE	602746	208002	lake	520	Aare	NA	0.31	NA	NA		NA	NA	NA	0	0	NA		0	0	NA	0	0	1.320	1	1
Morges	VD	525616	153773	river	450	Rhône		4	NA	mittelsteil		4	NA	NA	20.746	1	1	NA		0	0	128.351	1	1	NA	0	0
Mühlebachkanal	ZG	682359	228193	river	442	Reuss		6	NA	mittelsteil	NA		NA	NA	NA	NA	NA	NA		0	0	14.818	1	1	NA	0	0
Mülbach	SG	756908	219418	river	464	Alpenrhein		3	NA	flach	NA		NA	NA	0.000	1	0	NA		0	0	15.760	1	1	NA	0	0
Muota	SZ	689727	207362	river	443	Reuss		5	NA	flach		2	2	2	0.009	1	0	NA		0	0	NA	0	0	NA	0	0
Murg	TG	709547	269688	river	393	Rhein		6	NA	flach		2	3	2	4.110	1	1	NA		0	0	1285.430	1	1	77.280	1	1

Table S1. (cont.)

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish		DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
Murg	BE	629420	233600	river	421	Aare	5	NA	flach	3	NA	NA		3.229	1	0	NA	0	0	594.671	1	1	322.121	1	1
Murtensee	FR	569213	196431	lake	429	Aare	NA	23	NA	NA	NA	NA		NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
Nant du Pré Fleury	GE	490953	115194	river	350	Rhône	3	NA	steil	4	NA	NA		0.639	1	1	NA	0	0	2.501	1	1	NA	0	0
Nant du Pré Fleury	GE	491166	115272	river	364	Rhône	3	NA	steil	3	NA	NA		0.892	1	1	NA	0	0	NA	0	0	NA	0	0
Necker	SG	725714	249614	river	560	Rhein	6	NA	flach	NA	3	3		0.392	1	0	NA	0	0	392.064	1	1	NA	0	0
Neirigue	FR	566022	174867	river	649	Aare	4	NA	mittelsteil	4	NA	NA		NA	0	0	NA	0	0	22.091	1	1	2.359	1	1
Neuenburgersee	NE	557505	202529	lake	429	Aare	NA	217.9	NA	NA	NA	NA		NA	0	0	NA	0	0	NA	0	0	NA	0	0
Neuenburgersee	FR	554242	189237	lake	429	Aare	NA	217.9	NA	NA	NA	NA		0.559	1	1	NA	0	0	NA	0	0	NA	0	0
Neuenburgersee	VD	539007	184028	lake	429	Aare	NA	217.9	NA	NA	NA	NA		0.498	1	0	NA	0	0	26.073	1	1	NA	0	0
Neuenburgersee	NE	549305	193520	lake	429	Aare	NA	217.9	NA	NA	NA	NA		NA	0	0	NA	0	0	1.563	1	0	NA	0	0
Neuenburgersee	VD	538874	181993	lake	429	Aare	5	217.9	NA	NA	NA	NA		NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
Nidau-Büren-Kanal	BE	588258	219128	river	427	Aare	9	NA	flach	1	NA	NA		1.111	1	0	0.704	1	0	NA	0	0	NA	0	0
Obere Lorze	ZG	684785	226755	river	510	Reuss	6	NA	mittelsteil	NA	NA	NA		6.271	1	1	NA	0	0	313.505	1	1	NA	0	0
Orbe	VD	518682	173720	river	748	Aare	3	NA	flach	3	NA	NA		8.925	1	1	NA	0	0	164.596	1	1	NA	0	0
Orbe	VD	506132	160583	river	1014	Aare	3	NA	flach	3	NA	NA		NA	0	0	NA	0	0	14.497	1	1	4.942	1	1
Orbe	VD	505936	160405	river	1015	Aare	3	NA	flach	3	NA	NA		0.515	1	0	NA	0	0	NA	0	0	NA	0	0
Ova Cristansains	GR	786232	155867	lake	1712	Inn	NA	0.018	NA	NA	NA	NA		0.051	1	0	NA	0	0	10.006	1	1	NA	0	0
Pfaffern	AG	634570	239587	river	409	Aare	4	NA	mittelsteil	3	2	2		0.961	1	0	5.857	1	0	1152.665	1	1	539.548	1	1
Pfäffikersee	ZH	701467	246697	lake	540	Rhein	NA	3.3	NA	NA	NA	NA		NA	0	0	NA	0	0	78.04	1	1	NA	0	0
Plessur	GR	761229	188959	river	1013	Alpenrhein	6	NA	mittelsteil	4	NA	NA		NA	0	0	NA	0	0	1.146	1	0	NA	0	0
Poschiavino	GR	806160	127525	river	882	Adda	6	NA	steil	2	NA	NA		NA	0	0	NA	0	0	1.54	1	1	NA	0	0
Promenthouse	VD	509987	140076	river	391	Rhône	4	NA	mittelsteil	4	3	4		17.81	1	1	NA	0	0	604.883	1	1	NA	0	0
Raffoltersee	ZH	701303	275360	lake	428	Rhein	NA	0.02	NA	NA	NA	NA		NA	0	0	NA	0	0	NA	0	0	NA	0	0
Reppisch	ZH	680187	236660	river	650	Limmat	3	NA	flach	3	NA	NA		0	1	0	NA	0	0	NA	0	0	NA	0	0
Reuss	AG	659446	259182	river	376	Reuss	8	NA	flach	2	NA	NA		1.562	1	1	NA	0	0	53.721	1	1	NA	0	0
Reuss	UR	690341	192583	river	441	Reuss	6	NA	flach	1	NA	NA		NA	0	0	NA	0	0	NA	0	0	NA	0	0
Rhein	SG	766677	251322	river	410	Alpenrhein	8	NA	flach	1	NA	NA		0	1	0	NA	0	0	129.149	1	1	NA	0	0
Rhein	ZH	686928	273076	river	346	Rhein	8	NA	flach	3	NA	NA		NA	0	0	NA	0	0	NA	0	0	NA	0	0
Rhein	BS	611120	270155	river	245	Rhein	9	NA	flach	1	NA	NA		NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
Rhein	AG	625522	266846	river	274	Rhein	9	NA	NA	1	NA	NA		0.964	1	0	NA	0	0	27.856	1	1	NA	0	0
Rhein	SH	685699	270637	river	340	Rhein	8	NA	flach	3	NA	NA		0.029	1	1	NA	0	0	1.815	1	1	NA	0	0
Rhein	ZH	684035	267395	river	344	Rhein	8	NA	NA	3	NA	NA		2.163	1	1	NA	0	0	152.377	1	1	NA	0	0
Rhein	ZH	674971	268914	river	334	Rhein	8	NA	flach	3	NA	NA		1.605	1	0	NA	0	0	20.885	1	1	NA	0	0
Rheintaler Binnenkanal	SG	761794	245488	river	410	Alpenrhein	6	NA	flach	NA	NA	NA		0	1	0	NA	0	0	108.882	1	1	0.701	1	0
Rhône	VS	587640	116293	river	474	Rhône	7	NA	flach	2	NA	NA		0.049	1	0	NA	0	0	26.91	1	1	NA	0	0
Rhône	VS	639618	128789	river	661	Rhône	6	NA	flach	2	NA	NA		0.005	1	0	NA	0	0	22.861	1	1	NA	0	0
Rhone	VD	557661	133278	river	382	Rhône	7	NA	flach	2	NA	NA		0.182	1	0	NA	0	0	NA	0	0	NA	0	0
Rialo d'Origlio	TI	716540	101807	river	420	Ticino	3	NA	flach	1	NA	NA		316.747	1	1	NA	0	0	254.755	1	1	NA	0	0
Riedbach	ZH	677167	259985	river	420	Rhein	1	NA	NA	1	NA	NA		52.788	1	1	0.74	1	0	1134.685	1	1	9.452	1	1
Rigi Aa	SZ	683281	213064	river	428	Reuss	4	NA	mittelsteil	2	NA	NA		0.841	1	1	NA	0	0	1011.991	1	1	NA	0	0
Rom	GR	830619	167828	river	1245	Adige	4	NA	NA	3	NA	NA		NA	0	0	NA	0	0	0	1	1	NA	0	0
Ron	LU	671784	218329	river	411	Reuss	4	NA	flach	2	NA	NA		NA	0	0	NA	0	0	14.932	1	1	NA	0	0

Table S1. (cont.)

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
Ron	LU	663879	224777	river	465	Aare		3	NA	flach		3	3	2	0.043	1	1	0.826	1	0	1238.563	1	1	0.790	1	1
Rot	LU	642769	223301	river	511	Aare		4	NA	flach		2	NA	NA	NA	0	0	NA	0	0	444.458	1	1	NA	0	0
Rot	LU	630188	230487	river	451	Aare		4	NA	mittelsteil		3	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Rotbach	LU	669866	218886	river	412	Reuss		5	NA	flach		2	NA	NA	NA	0	0	NA	0	0	135.519	1	1	NA	0	0
Roulave	GE	488456	117890	river	384	Rhône		2	NA	mittelsteil	NA		NA	NA	5.199	1	1	NA	0	0	NA	0	0	NA	0	0
Ruisseau du Tabeillon	JU	583984	243402	river	485	Rhein		2	NA	mittelsteil	NA		NA	NA	86.741	1	1	NA	0	0	105.719	1	1	NA	0	0
Rumensee	ZH	687102	242645	lake	546	Limmat	NA	0.012	NA	NA		NA	NA	NA		0	0	NA	0	0	NA	0	0	NA	0	0
Saane	BE	581767	192213	river	486	Aare		7	NA	flach	NA		3	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
sans nom chez Praille	GE	488969	119865	river	405	Rhône		1	NA	mittelsteil	NA		NA	NA	NA	NA	NA	NA	0	0	0.026	1	0	0.037	1	0
Saumbach	ZH	679275	261579	river	410	Rhein	NA	NA	flach		4	NA	NA	NA		0	0	NA	0	0	1.858	1	1	NA	0	0
Schiffenensee	FR	577381	188465	lake	531	Aare	NA	4.25	NA	NA		NA	NA	NA		0	0	NA	0	0	174.976	1	1	NA	0	0
Schiffenensee	BE	581192	192230	lake	531	Aare	NA	4.25	NA	NA		NA	NA		2.268	1	1	NA	0	0	2764.289	1	1	NA	0	0
Schiltebach	SH	674868	279850	river	400	Rhein		4	NA	flach		2	NA	NA	0.003	1	0	NA	0	0	4.000	1	1	29.430	1	1
Schübelweiher	ZH	687339	242108	lake	521	Limmat	NA	0.015	NA	NA		NA	NA	NA		0	0	NA	0	0	2.918	1	0	NA	0	0
Seez	SG	744172	218588	river	450	Limmat		5	NA	flach	NA		NA	NA	0.480	1	1	NA	0	0	312.329	1	1	NA	0	0
Sempachersee	LU	653675	223763	lake	504	Aare	NA	113.6	NA	NA		NA	NA	NA		0	0	NA	0	0	21.062	1	1	NA	0	0
Sense	FR	589913	172490	river	927	Aare		5	NA	mittelsteil		4	NA	NA	NA	0	0	NA	0	0	3.776	1	1	NA	0	0
Sense	BE	589249	193617	river	520	Aare		7	NA	mittelsteil		2	3	NA	NA	0	0	NA	0	0	NA	0	0	NA	NA	NA
Sense	FR	589147	175499	river	826	Aare		6	NA	mittelsteil		4	NA	NA	NA	0	0	NA	0	0	1.323	1	0	NA	0	0
Sense	FR	591650	173890	river	913	Aare		6	NA	mittelsteil		4	NA	NA	NA	0	0	NA	0	0	47.753	1	1	NA	0	0
Sihl	ZH	681914	245145	river	380	Limmat		7	NA	flach		2	NA	NA	0.893	1	1	NA	0	0	14.466	1	1	25.766	1	1
Sihlsee	SZ	702056	218926	lake	889	Limmat	NA	10.85	NA	NA		NA	NA		0.327	1	0	NA	0	0	444.382	1	1	NA	0	0
Sihlsee	SZ	701134	222968	lake	889	Limmat	NA	10.85	NA	NA		NA	NA		0.910	1	1	NA	0	0	53.860	1	1	NA	0	0
Silberbach	SZ	702942	227690	river	469	Limmat		2	NA	steil		3	NA	NA	NA	0	0	NA	0	0	8.584	1	0	NA	0	0
Silvaplanersee	GR	781832	149565	lake	1795	Inn	NA	3.2	NA	NA		NA	NA	NA		0	0	NA	0	0	5.381	1	1	NA	0	0
Simmi	SG	753794	230458	river	440	Alpenrhein		4	NA	mittelsteil	NA		NA	NA	0.454	1	1	3.686	1	1	116.319	1	1	24.568	1	1
Sissle	AG	643439	263267	river	340	Rhein		5	NA	mittelsteil		2	3	NA	0.984	1	0	NA	0	0	611.653	1	1	115.596	1	1
Sitter	SG	738233	262849	river	475	Rhein		6	NA	flach		3	3	NA	0.164	1	0	NA	0	0	22.126	1	1	NA	0	0
Stadtbach	AG	664386	257771	river	381	Limmat		3	NA	NA		2	NA	NA	0.313	1	0	NA	0	0	14.019	1	1	NA	0	0
Steinenbach	SG	716133	230806	river	430	Limmat		5	NA	flach	NA		NA	NA	0.699	1	1	NA	0	0	90.822	1	1	NA	0	0
Steinhauser Waldweiher	ZG	679744	229377	lake	468	Reuss		1	0.013	NA	NA		NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Steinigerbach	GL	722180	203768	river	553	Limmat		3	NA	mittelsteil		2	NA	NA	NA	0	0	NA	0	0	67.962	1	1	NA	0	0
Suhre	AG	648704	247459	river	397	Aare		5	NA	flach		2	2	NA	5.258	1	1	NA	0	0	580.143	1	1	87.518	1	1
Suhre	LU	647217	233138	river	477	Aare		4	NA	flach		2	NA	NA	0.000	1	0	NA	0	0	45.782	1	1	0.517	1	0
Surb	AG	662345	268507	river	328	Aare		4	NA	mittelsteil		3	2	2	2.068	1	0	NA	0	0	181.077	1	1	173.494	1	1
Suze	BE	586889	221562	river	437	Aare		3	NA	mittelsteil		1	2	NA	0.949	1	1	NA	0	0	NA	0	0	NA	0	0
Talent	VD	538774	162788	river	648	Aare		4	NA	mittelsteil		4	NA	NA	NA	0	0	NA	0	0	47.929	1	1	0.003	1	0
Talent	VD	539458	164228	river	631	Aare		4	NA	mittelsteil		4	NA	NA	0.000	1	0	NA	0	0	16.175	1	1	NA	0	0
Talent	VD	534682	167559	river	555	Aare		4	NA	flach		4	NA	NA	0.001	1	0	NA	0	0	NA	0	0	NA	0	0
Tanklagerweiher	AG	662645	253284	lake	350	Reuss	NA	0.022	NA	NA		NA	NA		1.269	1	0	NA	0	0	12.610	1	1	NA	0	0
Taverna	FR	586209	187939	river	617	Aare		3	NA	mittelsteil	NA		NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	1	1
Thunersee	BE	627075	167706	lake	559	Aare	NA	48.4	NA	NA		NA	NA		22.311	1	1	NA	0	0	32.210	1	1	NA	0	0

Table S1. (cont.)

														A. astaci			*B. dendrobatidis*			*S. parasitica*			*T. bryosalmonae*		
name	canton	CH1903 E	CH1903 N	waterbody	elevation	catchment	Strahler order	lake size	slope	ecomorph	IBCH	fish	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD	DNA concentration	below LOD	above LOD
Thur	ZH	691264	272339	river	410	Rhein		7	NA	flach		2	1	NA	1.530	1	1	NA	0	0	65.172	1	1	NA	0	0
Thur	SG	724097	245986	river	440	Rhein		6	NA	flach	NA	NA	NA		0.378	1	0	NA	0	0	166.945	1	1	1.928	1	1
Thur	SG	725572	238604	river	610	Rhein		5	NA	flach	NA	NA	NA		0.232	1	0	NA	0	0	501.306	1	1	NA	0	0
Ticino	TI	713303	137171	river	289	Ticino		5	NA	mittelsteil		2	NA	NA	1.875	1	0	NA	0	0	188.689	1	1	NA	0	0
Ticino	TI	721850	118077	river	221	Ticino		6	NA	NA		2	NA	NA	1.156	1	0	NA	0	0	1908.981	1	1	NA	0	0
Torneresse	VD	577630	141252	river	1141	Aare		3	NA	steil		2	NA	NA	NA	0	0	NA	0	0	199.091	1	1	NA	0	0
Töss	ZH	691415	263817	river	388	Rhein		6	NA	flach		2	NA	NA	0.899	1	0	NA	0	0	10.718	1	0	NA	0	0
Töss	ZH	685830	265590	river	370	Rhein		6	NA	flach		3	4	NA	2.888	1	1	NA	0	0	43.167	1	1	51.276	1	1
Tümpel bei Gheid	ZH	675819	255739	lake	424	Limmat	NA	0.0035	NA	NA		NA	NA		1.351	1	0	NA	0	0	NA	0	0	NA	0	0
Umgehungsgew. Hardau	ZH	693243	263582	river	360	Rhein		6	NA	flach		1	NA	NA	0.008	1	1	NA	0	0	46.827	1	1	1.585	1	1
Untere Lorze	ZG	676182	227716	river	401	Reuss		6	NA	mittelsteil	NA		2	NA	0.041	1	0	NA	0	0	26.724	1	1	NA	0	0
Urnäsch	AR	742495	251584	river	591	Rhein		5	NA	mittelsteil		3	3	2	0.504	1	0	1.535	1	1	217.154	1	1	4.625	1	1
Vedeggio	TI	714220	96057	river	260	Ticino		5	NA	flach		2	NA	NA	4.308	1	1	NA	0	0	637.967	1	1	NA	0	0
Venoge	VD	531420	155571	river	386	Rhône		4	NA	flach		4	2	2	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Venoge	VD	531384	152974	river	378	Rhône		4	NA	flach		4	NA	NA	NA	0	0	NA	0	0	4.439	1	1	NA	0	0
Venoge	VD	530249	166844	river	451	Rhône		4	NA	mittelsteil		3	NA	NA	NA	0	0	NA	0	0	NA	0	0	NA	0	0
Venoge	VD	522318	164384	river	639	Rhône		3	NA	mittelsteil		4	NA	NA	0.854	1	1	NA	0	0	38.264	1	1	NA	0	0
Versoix	VD	499345	131227	river	463	Rhône		4	NA	flach		4	NA	NA	0.477	1	1	NA	0	0	NA	0	0	0.044	1	0
Versoix	GE	502042	125742	river	373	Rhône		4	NA	flach		1	NA	NA	NA	0	0	NA	0	0	0.483	1	1	NA	0	0
Versoix	GE	498448	129802	river	455	Rhône		4	NA	flach		2	NA	NA	NA	0	0	NA	0	0	135.347	1	1	8.408	1	1
Verzasca	TI	709785	115108	river	203	Ticino		6	NA	mittelsteil		4	NA	NA	1.653	1	0	NA	0	0	876.380	1	1	NA	0	0
Vierwaldstättersee	SZ	687197	206195	lake	434	Reuss	NA	113.6	NA	NA		NA	NA	NA		0	0	NA	0	0	NA	0	0	NA	0	0
Vierwaldstättersee	LU	668082	211561	lake	434	Reuss	NA	113.6	NA	NA		NA	NA	NA		0	0	NA	0	0	0.288	1	1	NA	0	0
Vorderrhein	GR	746476	186788	river	665	Alpenrhein		7	NA	flach		4	NA	NA	NA	0	0	NA	0	0	6.240	1	1	NA	0	0
Wägitaler Aa	SZ	708850	227574	river	421	Limmat		6	NA	mittelsteil		2	NA	NA	0.196	1	0	NA	0	0	1315.816	1	1	NA	0	0
Wägitalersee	SZ	711134	218116	lake	898	Limmat	NA	4.18	NA	NA		NA	NA	NA		0	0	NA	0	0	NA	0	0	NA	0	0
Waldbach	ZH	698716	259954	river	460	Rhein		2	NA	steil		3	NA	NA	1.144	1	0	NA	0	0	33.330	1	1	NA	0	0
Walensee	SG	725954	221730	lake	419	Limmat	NA	24.1	NA	NA		NA	NA	NA		0	0	NA	0	0	42.712	1	1	NA	0	0
Wattbach	ZH	677652	230615	river	450	Reuss		3	NA	mittelsteil		1	NA	NA	1.525	1	1	NA	0	0	296.114	1	1	6.560	1	1
Werdenberger Binnenkanal	SG	754844	230621	river	440	Alpenrhein		5	NA	flach	NA		NA	NA	0.135	1	0	NA	0	0	263.853	1	1	NA	0	0
Werdenberger Binnenkanal	SG	757345	236704	river	430	Alpenrhein		6	NA	flach	NA		NA	NA	0.609	1	1	NA	0	0	1079.504	1	1	NA	0	0
Wiese	BS	612862	269449	river	253	Rhein		4	NA	flach		2	NA	NA	25.292	1	1	NA	0	0	NA	0	0	NA	0	0
Wigger	LU	639905	231994	river	456	Aare		6	NA	mittelsteil		3	3	NA	0.082	1	1	NA	0	0	410.257	1	1	NA	0	0
Wigger	LU	642784	219673	river	543	Aare		6	NA	mittelsteil		2	NA	NA	NA	0	0	NA	0	0	50.670	1	1	1.401	1	1
Wutach	SH	677073	289701	river	460	Rhein		5	NA	flach		3	NA	NA	NA	0	0	NA	0	0	678.186	1	1	70.321	1	1
Zihl	NE	539166	182228	river	429	Aare		5	NA	flach	NA		4	NA	0.026	1	0	NA	0	0	8.015	1	1	NA	0	0
Zihl	VD	537515	180033	river	429	Aare		5	NA	flach	NA		4	NA	0.009	1	0	NA	0	0	18.053	1	1	NA	0	0
Zugersee	ZG	680882	217975	lake	416	Reuss	NA	38.3	NA	NA		NA	NA		3.641	1	1	NA	0	0	20.394	1	1	NA	0	0
Zugersee	ZG	680907	225147	lake	414	Reuss	NA	38.3	NA	NA		NA	NA		0.000	1	0	NA	0	0	NA	0	0	NA	0	0
Zugersee	ZG	680771	225217	lake	414	Reuss	NA	38.3	NA	NA		NA	NA		2.268	1	1	NA	0	0	427.244	1	1	NA	0	0
Zürichsee	SZ	702667	229369	lake	406	Limmat	NA	90.1	NA	NA		NA	NA	NA	NA	NA	NA	NA	0	0	NA	0	0	NA	0	0
Zürichsee	ZH	683755	246058	lake	404	Limmat	NA	90.1	NA	NA		NA	NA	NA		0	0	NA	0	0	1.654	1	0	NA	0	0

Table S2. Dilution series of Gblocks fragment for absolute quantification and determination of LOD (limit of detection) for *S. parasitica*. Standards 5 to 11 were run in 30, and standards 12 to 15 in 40 replicates. Mean Cq-values and detection rates are calculated from all replicates.

Standard	Dilution	copies μl^{-1}	nr. of replicates	mean Cq-values ± SD	% detection
5	5^-5	3.47E+05	30	20.99 ± 2.95	100
6	5^-6	6.93E+04	30	21.35 ± 0.13	100
7	5^-7	1.39E+04	30	23.58 ± 0.16	100
8	5^-8	2.77E+03	30	26.06 ± 0.64	100
9	5^-9	5.55E+02	30	28.52 ± 0.20	100
10	5^-10	1.11E+02	30	30.94 ± 0.30	100
11	5^-11	2.22E+01	30	32.95 ± 0.45	100
12	5^-12	4.44E+00	40	34.36 ± 0.94	100
13	5^-13	8.87E-01	40	35.75 ± 0.91	62.5
14	5^-14	1.77E-01	40	36.06 ± 1.13	27.5
15	5^-15	3.55E-02	40	36.12 ± 0.00	0.025

Table S3. Binomial generalised linear mixed effects model results of variables on pathogen detection in water.

		Aphanomyces astaci			*Batrachochytrium dendrobatidis*			*Saprolegnia parasitica*			*Tetracapsuloides bryosalmonae*		
independent variables	nr. of sites	χ^2	df	p - value	χ^2	df	p - value	χ^2	df	p - value	χ^2	df	p - value
waterbody type	280	5.773	1	0.016	< 0.001	1	0.993	23.650	1	**< 0.001***	9.815	1	0.002
elevation	280	3.329	1	0.068	0.076	1	0.783	0.670	1	0.413	1.973	1	0.160
lake surface area[1]	68	0.177	1	0.674	n.a.	n.a.	n.a.	14.668	1	**< 0.001***	0.200	1	0.655
Strahler order	212	0.106	1	0.744	0.650	1	0.420	0.063	1	0.803	3.512	1	0.061
slope category	199	3.280	2	0.194	0.943	2	0.624	4.322	2	0.115	5.427	2	0.066
ecomorphology	179	0.031	1	0.860	0.117	1	0.732	0.070	1	0.792	0.462	1	0.497
invertebrate community	47	0.002	1	0.963	0.341	1	0.559	0.312	1	0.576	0.030	1	0.863
fish community	23	1.638	1	0.201	0.647	1	0.421	0.063	1	0.803	1.200	1	0.273

[1]*B. dendrobatidis* was not detected in lakes, so the analysis is not applicable.

Table S4. List of occupancy models and model fit test results of the posterior Predictive Loss (PPLC) and the Watanabe-Akaike Information criterions (WAIC) computed with the eDNAOccupancy R package (Dorazio & Erickson 2018) and listed for each pathogen. Negative ΔPPLC or ΔWAIC mean better model fit. Waterbody = lakes or rivers, lake_size = lake surface area, river_order = Strahler order. Lake surface area and Strahler order had to be analysed from separate datasets because the R package does not allow for missing values in the covariate data. For occupancy probabilities on site Ψ(.), sample θ(.) and qPCR replicate p(.) level, only constant values are reported.

Aphanomyces astaci	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC
(Ψ(.)θ(.)p(.))	186.326	0.231	0.371	0.544	0.804		
(Ψ(waterbody)θ(.)p(.))	186.040	0.230		0.543	0.805	-0.286	-0.001
(Ψ(elevation)θ(.)p(.))	186.271	0.231		0.543	0.805	-0.056	0.000
(Ψ(elevation+waterbody)θ(.)p(.))	186.426	0.231		0.544	0.805	0.100	0.000
(Ψ(.)θ(waterbody)p(.))	186.802	0.231	0.370		0.804	0.476	0.000
(Ψ(.)θ(elevation)p(.))	186.892	0.232	0.373		0.804	0.566	0.001
(Ψ(.)θ(.)p(waterbody))	186.831	0.232	0.371	0.544		0.505	0.001
(Ψ(.)θ(.)p(elevation))	187.517	0.233				1.191	0.002
lakes							
(Ψ(.)θ(.)p(.))	33.777	0.165	0.214	0.646	0.710	0.000	0.000
(Ψ(lake_size)θ(.)p(.))	33.796	0.166		0.643	0.711	0.019	0.000
(Ψ(.)θ(lake_size)p(.))	33.763	0.164	0.215		0.709	-0.014	-0.001
(Ψ(elevation+lake_size)θ(.)p(.))	33.849	0.165		0.642	0.710	0.073	0.000
rivers							
(Ψ(.)θ(.)p(.))	152.445	0.256	0.427	0.524	0.820	0.000	0.000
(Ψ(river_order)θ(.)p(.))	152.829	0.256		0.522	0.820	0.384	0.001
(Ψ(.)θ(river_order)p(.))	152.848	0.256	0.430		0.820	0.403	0.001
(Ψ(elevation+river_order)θ(.)p(.))	152.646	0.257		0.522	0.819	0.201	0.001

Saprolegnia parasitica	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC
(Ψ(.)θ(.)p(.))	277.627	0.370	0.808	0.605	0.908	0.000	0.000
(Ψ(waterbody)θ(.)p(.))	277.529	0.369		0.604	0.908	-0.098	0.000
(Ψ(elevation)θ(.)p(.))	277.586	0.369		0.604	0.908	-0.040	-0.001
(Ψ(elevation+waterbody)θ(.)p(.))	277.540	0.369		0.602	0.908	-0.087	0.000
(Ψ(.)θ(waterbody)p(.))	277.553	0.369	0.810		0.908	-0.074	-0.001
(Ψ(.)θ(elevation)p(.))	277.630	0.369	0.808		0.908	0.003	-0.001
(Ψ(.)θ(elevation+waterbody)p(.))	277.481	0.369	0.810		0.908	-0.146	-0.001
(Ψ(.)θ(.)p(elevation))	277.986	0.371	0.808	0.605		0.359	0.001
lakes							
(Ψ(.)θ(.)p(.))	56.028	0.279	0.585	0.521	0.875	0.000	0.000
(Ψ(lake_size)θ(.)p(.))	56.026	0.280		0.513	0.875	-0.002	0.000
(Ψ(.)θ(lake_size)p(.))	56.144	0.281	0.597		0.875	0.116	0.002
(Ψ(elevation+lake_size)θ(.)p(.))	56.159	0.280		0.510	0.875	0.131	0.000
rivers							
(Ψ(.)θ(.)p(.))	152.445	0.256	0.427	0.524	0.820	0.000	0.000
(Ψ(river_order)θ(.)p(.))	152.829	0.256		0.522	0.820	0.384	0.001
(Ψ(.)θ(river_order)p(.))	152.848	0.256	0.430		0.820	0.403	0.001
(Ψ(elevation+river_order)θ(.)p(.))	152.646	0.257		0.522	0.819	0.201	0.001

Table S4. (cont.)

Tetracapsuloides bryosalmonae	PPLC	WAIC	Ψ(.)	θ(.)	p(.)	ΔPPLC	ΔWAIC
(Ψ(.)θ(.)p(.))	117.460	0.140	0.247	0.490	0.810	0.000	0.000
(Ψ(waterbody)θ(.)p(.))	117.631	0.140		0.491	0.810	0.170	0.000
(Ψ(altitude)θ(.)p(.))	117.715	0.140		0.489	0.809	0.255	0.000
(Ψ(altitude+waterbody)θ(.)p(.))	117.400	0.140		0.488	0.810	-0.060	0.000
(Ψ(.)θ(waterbody)p(.))	117.807	0.140	0.248		0.810	0.347	0.001
(Ψ(.)θ(altitude)p(.))	117.535	0.141	0.247		0.810	0.075	0.001
(Ψ(.)θ(altitude+waterbody)p(.))	117.647	0.140	0.249		0.810	0.186	0.000
(Ψ(.)θ(.)p(altitude))	118.349	0.142	0.247	0.491		0.889	0.002
rivers							
(Ψ(.)θ(.)p(.))	152.445	0.256	0.427	0.524	0.820	0.000	0.000
(Ψ(river_order)θ(.)p(.))	152.829	0.256		0.522	0.820	0.384	0.001
(Ψ(.)θ(river_order)p(.))	152.848	0.256	0.430		0.820	0.403	0.001
(Ψ(altitude+river_order)θ(.)p(.))	152.646	0.257		0.522	0.819	0.201	0.001

Table S5. Linear mixed effects model results of variables on pathogen DNA concentrations in water.

		Aphanomyces astaci			*Batrachochytrium dendrobatidis*			*Saprolegnia parasitica*			*Tetracapsuloides bryosalmonae*		
independent variables	nr. of sites	χ^2	df	p - value	χ^2	df	p - value	χ^2	df	p - value	χ^2	df	p - value
waterbody type	280	0.602	1	0.438	n.a.	1	n.a.	3.399	1	0.065	0.585	1	0.444
elevation	280	1.158	1	0.282	0.580	1	0.446	9.807	1	**0.002***	1.854	1	0.173
lake surface area[1]	68	0.433	1	0.511	n.a.	n.a.	n.a.	0.143	2	0.705	n.a.	n.a.	n.a.
Strahler order	212	0.964	1	0.326	2.428	1	0.119	2.060	1	0.151	0.858	1	0.354
slope category	199	2.855	2	0.240	0.775	1	0.379	5.479	2	0.065	0.529	1	0.467
ecomorphology	179	0.014	1	0.906	0.414	1	0.520	0.002	1	0.962	0.944	1	0.331
invertebrate community	47	1.446	1	0.229	0.518	1	0.472	0.938	1	0.333	1.167	1	0.280
fish community	23	0.759	1	0.384	n.a.	n.a.	n.a.	3.720	1	0.054	1.498	1	0.221

[1]*B. dendrobatidis* was not detected in lakes and *T. bryosalmonae* was detected in one lake only, so the analysis is not applicable.

[2]*B. dendrobatidis* was only found in sites with fish communities ranked as "very good", so the analysis is not applicable.

Table S6. Linear mixed effects model results of variables on pathogen DNA concentrations in water above the limit of quantification (LOQ).

		Aphanomyces astaci			*Saprolegnia parasitica*			*Tetracapsuloides bryosalmonae*		
independent variables	nr. of sites	χ^2	df	p - value	χ^2	df	p - value	χ^2	df	p - value
waterbody type[1]	280	0.875	1	0.350	1.336	1	0.248	n.a.	n.a.	n.a.
elevation	280	3.195	1	0.074	4.048	1	**0.044***	4.227	1	**0.040***
lake surface area[1]	68	20.773	1	**< 0.001***	0.211	1	0.646	n.a.	n.a.	n.a.
Strahler order	212	0.744	1	0.389	4.246	1	**0.039***	1.306	1	0.253
slope category	199	3.610	1	0.057	2.108	2	0.348	0.414	1	0.520
ecomorphology	179	0.002	1	0.969	0.019	1	0.890	4.016	1	**0.045***
invertebrate community	47	0.207	1	0.649	0.842	1	0.359	0.338	1	0.561
fish community	23	0.273	1	0.602	0.089	1	0.766	0.072	1	0.788

[1]*T. bryosalmonae* DNA concentrations above LOQ were only found in rivers, so the analysis is not applicable

Table S7. Binomial generalised linear mixed effects model results of variables on pairwise pathogen co-detection in water.

		Aphanomyces astaci - *Saprolegnia parasitica*			*Aphanomyces astaci* - *Tetracapsuloides bryosalmonae*			*Saprolegnia parasitica* - *Tetracapsuloides bryosalmonae*		
independent variables	nr. of sites	χ^2	df	p - value	χ^2	df	p - value	χ^2	df	p - value
waterbody type[1]	280	3.6872	1	0.05483	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
elevation	280	3.1426	1	0.07627	2.9115	1	0.08795	4.1644	1	**0.04128***
lake surface area[1]	68	0.3389	1	0.5605	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Strahler order	212	0.0247	1	0.8752	0.275	1	0.59999	2.1066		0.14667
slope category	199	5.1117	2	0.07763	1.5258	2	0.46631	3.6757	2	0.15916
ecomorphology	179	0.0845	1	0.7713	0.2537	1	0.6145	0.2527	1	0.61517

[1]*T. bryosalmonae* was not co-detected with other pathogens in lakes, so the analysis is not applicable.

Table S8. Presence / absence (1 / 0) of T. bryosalmonae in eDNA water samples and fish of rivers where both datasets were available. Coordinates are given for eDNA water sampling sites and fish sampling sites and the date of fish collection is listed.

waterbody name	eDNA CH1903 E	eDNA CH1903 N	fish CH1903 E	fish CH1903 N	fish date	eDNA result	fish result
Aabach	654895	251775	656000	248000	13.09.2000	1	1
Aabach	714846	231225	714860	231220	29.09.2004	1	1
Aabach	695479	244545	696005	244765	03.10.2006	0	1
Albula	755266	174496	755000	175000	05.09.2000	0	0
Allaine	568089	260720	568089	260720	16.09.2006	1	1
Allondon	489615	115922	490000	116000	19.09.2000	1	1
Alpenrhein	760592	200693	760000	196000	14.08.2005	0	0
Areuse	553724	200788	554704	200247	28.09.2005	0	0
Aubonne	520742	147728	520259	146511	09.09.2000	0	0
Birs	612790	256385	612747	261505	31.10.2006	1	1
Birsig	609092	262535	610052	264179	06.09.2002	1	1
Bodensee	755558	260697	756800	260900	20.10.1999	0	0
Broye	566155	191903	561560	185539	05.10.2004	0	1
Doubs	572586	244466	577041	245481	21.09.2005	0	1
Dünnern	629548	241889	628131	241370	21.10.2005	0	0
Eaux-Froides	489598	115884	490000	116000	09.10.2005	0	0
Emme	609479	225479	610692	227660	23.08.2002	0	1
Ergolz	629884	256865	630710	257172	06.09.2002	1	1
Erveratte	577822	252739	577840	252732	11.10.2004	1	1
Frenke	623169	258926	622400	257744	24.10.2006	0	0
Furtbach	669576	254667	668821	254514	30.08.2006	1	1
Glâne	562639	173073	562300	172700	05.10.2004	1	1
Goldach	752257	259961	752260	260000	27.09.2000	0	0
Gulantschi	611482	128739	611595	128623	18.10.2004	0	0
Jona	711711	237782	711787	237868	17.10.2000	1	1
Jonen	671265	238823	676700	237300	14.09.2000	1	1
Kander	619088	163645	617020	160710	21.10.2003	0	0
Kempt	695426	258339	695530	258747	31.10.2000	1	1
Kleine Emme	661952	211537	661945	211498	19.10.2004	0	0
La Coeuvatte	573527	259819	573515	259814	11.10.2004	below LOD	1
Landquart	766508	205009	778000	198000	05.09.2000	0	0
Laveggio	719256	84526	719265	84543	05.10.2000	1	1
Limmat	672733	252780	671118	253543	31.10.2000	0	1
Linth-Kanal	719562	225201	717000	228000	27.09.2000	0	0
Littibach	683697	228998	683625	229375	11.10.2004	0	1
Luthern	640607	226452	640620	226539	20.10.2004	0	1
Lützel	602010	252773	602877	251876	21.05.2004	1	1
Mentue	545386	180937	545460	181100	07.09.2000	0	1
Mettlenbach	697038	240610	696459	238617	17.10.2000	0	1
Moesa	732612	123462	733000	126000	05.09.2000	0	0
Mönchaltorfer Aa	696394	241463	696395	241480	03.10.2006	0	1

Table S8. (cont.)

waterbody name	eDNA x	eDNA y	fish x	fish y	fish date	eDNA result	fish result
Mülbach	756908	219418	756920	218300	29.09.2004	0	0
Murg	629420	233600	629660	232380	19.08.2003	1	1
Murg	709547	269688	710000	269000	18.09.2000	1	1
Necker	725714	249614	725950	249625	12.09.2006	0	0
Neirigue	566022	174867	567150	175650	05.10.2004	1	1
Obere Lorze	684785	226755	683100	228600	04.10.2005	0	1
Orbe	518682	173720	517000	173000	17.08.2005	0	0
Orbe	506132	160583	505947	160413	07.09.2000	1	1
Ova Cristansains	786232	155867	786155	155631	31.08.2006	0	1
Pfaffern	634570	239587	635000	240000	13.09.2000	1	1
Plessur	761229	188959	771056	182859	05.09.2000	0	0
Poschiavino	806160	127525	803000	130000	05.09.2000	0	0
Reuss	659446	259182	673994	223426	21.09.2001	0	1
Rhein	625522	266846	620622	265408	06.09.2002	0	1
Rheintaler BK	761794	245488	761780	245400	07.10.2005	below LOD	1
Rigi Aa	683281	213064	682600	213550	20.10.2004	0	0
Ron	663879	224777	663540	225530	30.09.1999	1	0
Rot	630188	230487	630217	230307	19.10.2004	0	1
Rotbach	669866	218886	670089	219028	19.10.2004	0	1
R. d. Tabeillon	583984	243402	584027	243407	11.10.2004	0	1
Sense	589147	175499	590000	178000	04.09.2000	0	0
Sihl	681914	245145	686700	230300	15.10.2004	1	1
Simmi	753794	230458	754000	230730	29.09.2004	1	1
Sitter	738233	262849	744800	255400	27.09.2000	0	1
Steinenbach	716133	230806	717000	231000	27.09.2000	0	0
Suhre	648704	247459	649000	248000	13.09.2000	1	1
Surb	662345	268507	667000	264000	13.09.2000	1	1
Thur	725572	238604	725570	238580	16.08.2005	0	1
Ticino	713303	137171	715740	135425	05.10.2000	0	0
Töss	691415	263817	691000	263900	28.07.2005	0	1
Vedeggio	714220	96057	714161	95941	08.10.2004	0	1
Venoge	531420	155571	530500	156200	05.08.2003	0	0
Versoix	502042	125742	501000	126000	19.09.2000	0	1
Versoix	499345	131227	499353	131231	16.09.2004	below LOD	1
Vorderrhein	746476	186788	733830	181570	27.10.1998	0	0
Werdenberger BK	757345	236704	754571	228600	27.09.2000	0	0
Wigger	639905	231994	639825	232170	20.10.2004	0	1
Wigger	642784	219673	642841	219888	20.10.2004	1	1

Reference

Dorazio RM, Erickson RA (2018) Ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380

Chapter 4

Synthesis: remaining challenges and opportunities

The central aim of this thesis was to develop, test and apply an eDNA-based method and workflow for the detection of aquatic wildlife pathogens in water. The questions to answer now are if this eDNA-based method can reliably detect pathogens in water and, if yes, how it could best be implemented as an appropriate tool contributing to disease management. In this chapter I discuss implications of this work regarding these questions, list possible ways for improvement and point out future challenges and opportunities.

Dealing with imperfect detection

Both the experiments in Chapter 1 and the comparative study in Chapter 2 revealed limitations of the method, i.e. imperfect detection of *Batrachochytrium dendrobatidis* and *Tetracapsuloides bryosalmonae* spiked into water tanks and of *Aphanomyces astaci* at sites where infected crayfish were captured. With the increasing amount of eDNA studies being conducted, assessment of the reliability of detection and reduction of errors has become a central aspect in the field. In the following sections I discuss specific aspects influencing pathogen detection and possible improvements of sampling methodology and design, as well as sample processing.

Water sample volumes, filter type and storage

Detection rates could be influenced by water volume, filter type and pore size, and filter storage, amongst others. I took large water samples of 5 L to balance maximal capture of pathogen spores with reasonable filtration time. The effect of volume on detection rates was not tested in this thesis and is generally understudied in the eDNA literature (Sepulveda et al. 2019). Mächler et al. (2016) did not observe significant differences in detection rates of macroinvertebrates in samples with volumes of 250 ml to 2 L, and Wittwer et al. (2018a) did not observe differences between volumes of 10 L and 100 L on *A. astaci* detection, although different techniques were used. On the other hand, Sepulveda et al. (2019) experienced higher bull trout (*Salvelinus confluentus*) detection rates in samples of 1500 and 3000 L than 4 L. However, such large volumes are generally not feasible and experience a higher accumulation of PCR-inhibitory compounds in the DNA extractions (Lance & Guan 2020). Therefore, larger volumes are only recommended if further processing steps reduce the effect

of inhibition and if the required sampling effort and time do not exceed reasonable thresholds. However, decisions about sampling volumes can only be taken in conjunction with filter pore size. Small pore sizes capture smaller DNA particles but are more prone to clogging, creating a trade-off between increased water volume and capture of smaller DNA particles. I used glass fibre filters with an average pore size of 1 µm, which is small enough to capture zoospores of targeted pathogens, but extracellular DNA particles might not be captured. Filters did occasionally clog before the desired 5 L could be filtered, which could warrant usage of larger pore sizes for certain habitats. Glass fibre filters used in this study have been utilised successfully in eDNA studies before (e.g. Eichmiller et al. 2016, Lacoursière-Roussel et al. 2016, Spens et al. 2017). However, glass fibre filters are absorbent and can retain water and lysate containing DNA at the beginning of DNA extraction, potentially decreasing target DNA yield.

Furthermore, I observed that clogging of filters led to varying levels of water retention by the filter, which could have contributed to the observed variation and decreased yield caused by filters described in Chapter 1. Testing for improved DNA yield with other filter types than glass fibre and controlling water retention of filters, e.g. through drying of the filters, would therefore be interesting as a measure for increasing the consistency of results.

The collected samples were for the most part stored in ice, before being frozen at - 80°C in the lab, except during trips lasting several days, when samples were stored in a dry shipper cooled with liquid nitrogen prior to the trips. However, temperatures provided by ice do not completely stop degradation processes. Other preservation techniques have been applied to eDNA samples, like the addition of preservative solutions such as buffers (Renshaw et al. 2015) and ethanol (Minamoto et al. 2016), or by drying media, such as silica beads (Carim et al. 2016, Rusch et al. 2020). The addition of preservative solutions, however, affects next processing steps and might limit the choice for DNA extraction methods. Silica beads desiccate the filter to preserve the DNA (Bakker et al. 2017) which can then be transported without the need for cooling, an important aspect under challenging field conditions. It would be interesting to test the stability of the PowerWater DNA extraction kit lysis buffer for DNA transportation or the usage of silica beads and how storage affects detection rates. Water sample volumes, filter type and pore size, and filter storage, in combination with DNA extraction methods that differ in their effectiveness at reducing PCR inhibition, need to be carefully considered and potentially re-considered in future studies (Djurhuus et al. 2017).

DNA amplification methods and qPCR reaction volumes

Quantitative real-time PCR is considered more sensitive than conventional endpoint PCR (e.g. Helps et al. 2001, Borg et al. 2003, Emery et al. 2004, but see Bastien et al. 2008 for a contrary view) and hydrolysis probes further ensure specificity. For these reasons, qPCR has been extensively and successfully applied in eDNA studies and could be considered standard practice for species-specific eDNA-based surveys. Nevertheless, the usage of digital droplet PCR (ddPCR) has steadily increased and costs decreased in the last years. The advantage of ddPCR is that it omits the need for standards to quantify DNA concentrations, and that it is less influenced by inhibition (Sedlak et al. 2014, Hunter et al. 2017). Indeed, higher detection rates compared to qPCR have been observed in some studies (Doi et al. 2015, Wood et al. 2019). Digital PCR would therefore be a valid option for future species-specific pathogen detection attempts.

Many studies using qPCR to detect pathogens in water samples use reaction volumes of 20 or 25 µl (e.g. Boyle et al. 2004, Vrålstad et al. 2009, Rocchi et al. 2017). We reduced the reaction volume to 10 µl following Fontes et al (2017) to ensure all DNA extracts could be analysed for all pathogens and the internal positive control (IPC). However, this could have introduced increased variability in quantification and reduced the volume of sample analysed for each pathogen, i.e. instead of 5 µl only 2.5 µl of the target DNA were added per extraction, which could increase uncertainty in detection rate (Mächler et al. 2016). An increase in replicates could also have given more certainty (Ficetola et al. 2015), though we deemed three technical replicates to be an appropriate compromise between replication and costs.

Number and location of samples

Sampling consisted of a negative control sample of 5 L of purified clean water and three 5 L water samples per site, and in case of filter clogging, a maximum of six water samples of lower volume. For this, a single spot along the river or lake shore was chosen for placing the pump close enough to the water for the tubing to reach the water, but far enough that the pump did not directly contact the water. The number of samples was thought to be a good compromise between knowledge gained and effort and cost. However, according to occupancy modelling results (Dorazio and Erikson 2018), the number of samples per site in Chapters 2 and 3, or per tank in Chapter 1, would have needed to be even higher to reach detection probabilities of 95 % and above. In a study by Chestnut et al. (2014), *B. dendrobatidis* was found to be reliably detected if 4 – 5 water samples were taken from ponds

and wetlands. Increasing the number of samples per sampling site could therefore reduce false negatives but will also increase effort and cost of eDNA pathogen surveys. In Chapter 1, non-homogeneous distribution of zoospores in the water was identified as a potential main driver of inconsistent detections in water tanks of only 20 L of volume. Heterogeneous distribution is expected to be more pronounced in more complex natural aquatic systems, such as rivers or lakes. Therefore, in addition to, or instead of, increasing the number of samples, samples could be taken from different positions of a sampling site (Goldberg et al. 2018). This sampling design would consider heterogeneous distribution of pathogen zoospores in water without increasing the number of samples and with little extra effort. Schmidt et al. (2013) carry this argument further by stating that sampling more sites less extensively, i.e. taking fewer samples per location but more from different locations, would improve results in species distribution surveys if occupancy models are applied to quantify detection error.

Occupancy models for measuring uncertainty

When detection probabilities are less than one, models that estimate site occupancy rates can help accounting for missed observations (MacKenzie et al. 2002) and are therefore well suited for eDNA surveys (Schmidt et al. 2013). Hierarchical, or multiscale occupancy models (Nichols et al. 2008) provide detection estimates for multiple levels of the sampling process, i.e. for sampling site, water sample and qPCR replicate level. We used an R package developed by Dorazio and Erikson (2018), for fitting multiscale occupancy models using Bayesian Markov chain Monte Carlo (MCMC) methods to our data. Covariates, such as pathogen zoospore concentrations or environmental factors, could be included in the models and tested for their effect on occupancy probabilities on all three levels, though not many effects were found in the data. In all three chapters, occupancy probabilities were lower on sample level than on qPCR replicate level, since in many cases, only one or two out of three water samples per site tested positive for a pathogen. This indicates that increasing the number of samples might be more effective for reducing false negative results than increasing the number of qPCR replicates. Therefore, accounting for uncertainty on all levels of pathogen surveys could help pinpoint the areas whose improvement would decrease detection errors the most and thus help to develop efficient and reliable sampling designs in pathogen monitoring (McClintock et al. 2010).

Survey timing

Water samples in Chapters 2 and 3 were collected during summer, i.e. from May to October. This time period was chosen since host species are generally more active and, therefore, shedding more pathogens when water temperatures are warm. However, host species show strong temporal dynamics during the growing season, which can heavily affect pathogen loads in water. Hallett et al. (2012) measured highest spore levels of *Ceratonova shasta*, a myxosoporean parasite of salmonid fish, in river water in early summer. *Ranavirus* and *B. dendrobatidis* concentrations in pond water were observed to be closely linked to host developmental stages, rising when anuran hosts reach later tadpole stages (Hall et al. 2018, Julian et al. 2019, Miaud et al. 2019). Frog development in ponds is often synchronous for a large number of individuals and also depends on environmental factors such as water temperature. As discussed in Chapter 2, crayfish experience highest degrees of activity during the mating season in September and October in our latitudes and *A. astaci* concentrations have been shown to be highest during that period (Wittwer et al. 2018b). Interestingly, *T. bryosalmonae* experiences two periods of peak spore release, the first in May / June due to bryozoans shedding spores, and the second in September / October when infections have matured in fish (Hartikainen & Okamura 2015) A more temporally focused approach to eDNA-based surveillance closely linked to periods of highest pathogen abundance in water could therefore further improve detection reliability.

Remaining challenges and future avenues

This section discusses aspects deemed important for progress in the field of eDNA-based methods for pathogen detection in water. I focus on targeted detection of specific pathogens by qPCR. Several other topics, such as improvement of reference databases and application of targeted metabarcoding methods, are of equal importance but not discussed here.

Defining an appropriate limit of detection criterium for eDNA studies

The limit of detection (LOD) is an important criterium for determining the assay sensitivity of a qPCR assay (Bustin et al. 2009). It is described as the weakest signal strength that can be distinguished from the instrumental background noise or the lowest DNA concentrations that are reliably detected but not quantified (Burns & Valdivia 2008). The widely applied standard definition of the LOD is the DNA concentration at which the assay detects the presence of the target DNA at least 95 % of the time (Bustin et al. 2009). DNA concentrations below the LOD are then usually defined as negative. However, this can be problematic for

eDNA samples that often contain very low DNA concentrations in the vicinity of the LOD, which can cause underestimation of detection when many low-level detections are labelled as negative. Especially when dealing with pathogens that pose a high risk to endangered wildlife, such as the crayfish plague, false negatives are a concern. We therefore took a less stringent and more permissive approach to the LOD: for all assays it was defined as the first dilution of the standard curve with detection rate lower than 100% which happened to be the same dilution for all pathogen assays, with detection rates of 62.5 % for *A. astaci* and *S. parasitica* and 50 % for *B. dendrobatidis* and *T. bryosalmonae*. Klymus et al. (2019) propose to use the standard 95 % detection LOD definition as a property of the qPCR assay for comparison to other assays, but not for categorising eDNA samples according to their DNA concentrations. They argue that detection below LOD should be considered as true positive detection, given more permissive criteria, i.e. Cq-value ≥ 40, uniform curve morphology and no amplification in negative template controls (Klymus et al. 2019). Nevertheless, a generally accepted definition of LOD, or an alternative cut-off criterium, for eDNA studies is still lacking, but would be extremely useful.

Deeper understanding of underlying processes

Due to the relative novelty of the eDNA approach, especially for wildlife pathogens, researchers have focused on developing detection assays and exploring capabilities of these methods for pathogen detection. For future development of the field, the focus should shift from showing that it works to studying how and why it works (or not, sometimes), i.e. conducting assessments of performance, reliability and limitations of eDNA-based methods for pathogen detection. Lacoursière-Roussel and Deiner (2019) make a strong statement: "Without the fundamental knowledge of what eDNA is and how it interacts with its surroundings, an accurate inference that a species was present in a place and time remains a challenge." The authors call for more research on eDNA properties and dynamics. This would then enable us to optimise sampling schemes by minimising chances of errors, i.e. false negative and false positive detections, learn how to treat imperfect detection, and interpret results quantitively, perhaps even estimate pathogen loads at host population level and assess the risk of exposure and ultimately, disease. However, the factors and processes affecting behaviour of eDNA in the environment are still only partly described (Cristescu & Hebert 2018). Nevertheless, the effect of UV light, temperature and acidity on eDNA degradation has been studied in laboratory experiments (Strickler et al. 2015, Mächler et al. 2018). Movement of eDNA has been noted to be complicated and partly unpredictable in

experimental stream facilities (Shogren et al. 2017). In the field, Goldberg et al. (2018) observed lower amphibian eDNA detection rates calculated with occupancy models in wetlands with lower pH and Song et al (2017) discovered that flow reversals were responsible for carp eDNA detection patterns in the Chicago Area Waterway System. While pondering the reasons and implications of the results showed in Chapter 1 and 2, I started thinking about how the zoospores might move within the water column and that a myriad of factors might influence that movement. The literature on zoospore behaviour in water is scarce and has mostly focused on spore shedding from hosts (e.g. Reeder et al. 2012, Makkonen et al. 2013, Svoboda et al. 2013, Maguire et al. 2016). As with conventional monitoring methods, pathogen biology and behaviour have mostly been studied within the explicit context of the host, i.e. focusing on infectivity, virulence and transmission rates, and not what happens to the pathogen while between hosts. In Chapter 1, we hypothesised that heterogeneous distribution of zoospores in the tanks is a cause for inconsistent detection and variability in DNA concentrations, while in Chapter 2, non-detection of *A. astaci* in water samples from sites where infected crayfish were found, was deemed to be due to low spore shedding of asymptomatic hosts. However, the exact processes and factors causing these patterns in detection are unknown.

Experiments in controlled environments, such as aquaria, help disentangle these processes (Chapter 1, Makkonen et al. 2013, Svoboda et al. 2013). The amount of target organisms, e.g. as number of individuals or biomass, their life stage, water properties like temperature and pH, and many other potential factors influencing detection can be manipulated in such facilities. However, the flexibility of manipulation comes at the cost of realism and intercorrelations discovered in an aquarium or mesocosm might not hold for the ultimately more complex system of the real world. Therefore, field validations will also be needed. The performance of eDNA-based methods has been compared extensively to conventional methods in field settings (e.g. Jane et al. 2015, Wilcox et al. 2016, Hinlo et al. 2017, Eiler et al. 2018). These comparisons can give valuable insights into how host densities and pathogen prevalence in hosts can influence pathogen detection in water, as seen in Chapter 2. While identifying the exact processes leading to observed patterns in field settings is difficult, these observations can guide experimental designs with the goal to unravel underlying processes. Furthermore, with increasing empirical and experimental knowledge models could be built that predict important factors of pathogen persistence and behaviour in the environment (Carraro et al. 2017, 2018, Brunner & Yarber 2018) which can help inform eDNA-based pathogen surveys.

Detection of multiple pathogen and host species

Environmental DNA-based techniques create the opportunity for monitoring multiple pathogen species simultaneously across taxonomic boundaries and with minimal extra effort. While Chapter 3 shows the feasibility of surveys targeting several pathogens, it also presented many questions and potential for refinement. Since the occurrence of pathogens is closely linked to their host's, pathogen combinations are limited to those having hosts sharing the same habitats and similar seasonal dynamics. For instance, *A. astaci* and *T. bryosalmonae* hosts, i.e. crayfish and fish, such as trout, have overlapping habitats and even periods of highest pathogen concentrations in water in late summer, and could therefore be effectively combined in surveys. *B. dendrobatidis*, however, as shown in Chapter 3, seems to require different survey strategies. While amphibians have overlapping habitats with crayfish and fish, many species mostly appear in small, sometimes ephemeral, waterbodies during short time periods of the year. For *B. dendrobatidis* surveys the focus would therefore need to shift to small waterbodies and ideally be conducted earlier in the year, coinciding with amphibian mating period and tadpole development.

Researchers studying the occurrence of pathogens in water are faced with the additional layer of complexity arising from hosts. The effect of season on pathogen detection reliability and its close link to host phenology was discussed in all three Chapters and indicates that knowledge about host biology and behaviour is crucial for successful pathogen surveys. While this thesis focused on eDNA-based detection of pathogens only, simultaneous detection of both pathogen and host from eDNA can add valuable additional information to pathogen surveys. *Aphanomyces astaci* detection in water has been coupled to crayfish host eDNA detection (Robinson et al. 2018, Strand et al. 2019, Rusch et al. 2020) and a multiplex assay for the detection of *T. bryosalmonae* and its bryozoan host *Fredericella sultana* was developed and applied in Swiss rivers (Fontes et al. 2017, Carraro et al. 2018). Rusch et al. (2018) separately tested water samples for monogenean parasite *Gyrodactylus salaris* and fish hosts *Salmo salar* and *Oncorhynchus mykiss*. Host-parasite detection could be widened by including other host and pathogen species, if similar habitat requirements and seasonality warrant it. Multi-species eDNA surveys create the opportunity to receive valuable insights into host-parasite communities.

Closing the science-practice gap

The research field evolving around eDNA is of applied nature. The final goal of many eDNA studies is to show that eDNA-based techniques are a valid choice for species or disease

monitoring. However, for research results to be applied in practice scientists need to closely collaborate with managers and effective communication is required (Mosher et al. 2020). An intriguing approach to make eDNA-based methods more accessible to managers has been taken by Sepulveda et al (2020). The authors discuss and confirm the maturity of eDNA methods according to legal standards and conclude that decision-support frameworks are needed for integrating the uncertainty of eDNA-based methods into management. A manager reading Chapter 1 and 2 of this thesis might become doubtful of the eDNA method due to the ambiguous results. To remove uncertainty about reliability of eDNA-based methods, clear understanding of the method and its associated risks of erroneous results by all involved parties is therefore important when planning eDNA-based surveys. The tolerance for detection errors might depend on the survey goal, i.e. disease outbreak risk surveys would have low tolerance for false negative detections, while surveys investigating distribution of a less serious pathogen might be more permissive (Mosher et al. 2020). If the quantified risk of error is still deemed too high, eDNA-based surveillance could be coupled with conventional methods in case of ambiguous results (Lahoz-Monfort et al. 2016). Environmental DNA techniques increase options for creating disease surveillance schemes tailored to specific goals. The crucial step is now to bring together all involved stakeholders for the application and implementation of eDNA-based disease monitoring and species surveillance in general.

Conclusion

Raising the question once more, whether the present thesis can answer the question if eDNA-based methods offer a reliable method for pathogen detection in water, my answer would be a tentative yes. Chapter 3 showed that eDNA methods can be used for surveys of multiple pathogens, which would have been vastly more laborious to achieve with conventional disease survey methods. Nevertheless, results from Chapter 1 and 2 show clear limitations of the methods that need to be considered and addressed for future endeavours. Possible approaches for reducing imperfect detection are changes in water sample volume, filter type and field storage, qPCR reaction volumes and number of replicates. Further, and most importantly, sampling design, i.e. the number and location of samples, and timing of the survey, could improve reliability of detection. Since detection will never be perfect, occupancy models offer a valuable tool for quantifying detection errors and therefore uncertainty. For further optimisation of sampling methodology and design, shedding more

light on the underlying processes, such as zoospore behaviour in the water, the interplay between pathogen abundance in water, host densities and infection prevalence and seasonal dynamics, is required. Further, the potential of eDNA-based methods for surveillance of multiple pathogens and their hosts across taxonomic boundaries is still largely untapped and should be investigated further. Lastly, eDNA methods and the present thesis have an applied purpose and should therefore be accessible to managers. This requires clear communication of expectations and risks of eDNA-based detection and close collaboration with all the involved stakeholders. To conclude, I deem eDNA-based methods for the direct detection of pathogens in water as appropriate for implementation in pathogen surveillance. While limits of the method have become apparent in this thesis calling for further optimisation, no conventional method offers the same degree of flexibility and modularity for detecting multiple pathogens, with many possible extensions when applied along with host detection.

Literature cited

Bakker J, Wangensteen OS, Chapman DD, Boussarie G, Buddo D, Guttridge TL, Hertler H, Mouillot D, Vigliola L, Mariani S (2017) Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact. Sci Rep 7:1–11

Bastien P, Procop GW, Reischl U (2008) Quantitative real-time PCR is not more sensitive than ‘ conventional’ PCR. J Clin Microbiol 46:1897–1900

Borg I, Rohde G, Löseke S, Bittscheidt J, Schultze-Werninghaus G, Stephan V, Bufe A (2003) Evaluation of a quantitative real-time PCR for the detection of respiratory syncytial virus in pulmonary diseases. Eur Respir J 21:944–951

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60:141–148

Brunner JL, Yarber CM (2018) Evaluating the importance of environmental persistence for Ranavirus transmission and epidemiology, 1st ed. Elsevier Inc.

Burns M, Valdivia H (2008) Modelling the limit of detection in real-time quantitative PCR. Eur Food Res Technol 226:1513–1524

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009) The MIQE guidelines:

minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Carim KJ, McKelvey KS, Young MK, Wilcox TM, Schwartz MK (2016) A protocol for collecting environmental DNA samples from streams. USDA For Serv - Gen Tech Rep RMRS-GTR 2016:1–18

Carraro L, Bertuzzo E, Mari L, Fontes I, Hartikainen H, Strepparava N, Schmidt-Posthaus H, Wahli T, Jokela J, Gatto M, Rinaldo A (2017) Integrated field, laboratory, and theoretical study of PKD spread in a Swiss prealpine river. Proc Natl Acad Sci USA 114:11992–11997

Carraro L, Hartikainen H, Jokela J, Bertuzzo E, Rinaldo A (2018) Estimating species distribution and abundance in river networks using environmental DNA. Proc Natl Acad Sci USA 115:11724–11729

Chestnut T, Anderson C, Popa R, Blaustein AR, Voytek M, Olson DH, Kirshtein J (2014) Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. PLoS One 9

Cristescu ME, Hebert PDN (2018) Uses and misuses of environmental DNA in biodiversity science and conservation. Annu Rev Ecol Evol Syst 49:209–230

Djurhuus A, Port J, Closek CJ, Yamahara KM, Romero-Maraccini O, Walz KR, Goldsmith DB, Michisaki R, Breitbart M, Boehm AB, Chavez FP (2017) Evaluation of filtration and DNA extraction methods for environmental DNA biodiversity assessments across multiple trophic levels. Front Mar Sci 4:314

Doi H, Uchii K, Takahara T, Matsuhashi S, Yamanaka H, Minamoto T (2015) Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PLoS One 10:1–11

Dorazio RM, Erickson RA (2018) Ednaoccupancy: An r package for multiscale occupancy modelling of environmental DNA data. Mol Ecol Resour 18:368–380

Eichmiller JJ, Miller LM, Sorensen PW (2016) Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. Mol Ecol Resour 16:56–68

Eiler A, Löfgren A, Hjerne O, Nordén S, Saetre P (2018) Environmental DNA (eDNA)

detects the pool frog (*Pelophylax lessonae*) at times when traditional monitoring methods are insensitive. Sci Rep 8:1–9

Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, Tong S, Cook BT, Holloway BP, Mccaustland KA, Rota PA, Bankamp B, Lowe LE, Ksiazek TG, Bellini WJ, Anderson LJ (2004) Real-time reverse transcription – polymerase chain reaction assay for SARS-associated Coronavirus. Emerg Infect Dis 10:311–316

Ficetola GF, Pansu J, Bonin A, Coissac E, Giguet-Covex C, De Barba M, Gielly L, Lopes CM, Boyer F, Pompanon F, Rayé G, Taberlet P (2015) Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. Mol Ecol Resour 15:543–556

Fontes I, Hartikainen H, Holland JW, Secombes CJ, Okamura B (2017) *Tetracapsuloides bryosalmonae* abundance in river water. Dis Aquat Org 124:145–157

Goldberg CS, Strickler KM, Fremier AK (2018) Degradation and dispersion limit environmental DNA detection of rare amphibians in wetlands: increasing efficacy of sampling designs. Sci Total Environ 633:695–703

Hall EM, Goldberg CS, Brunner JL, Crespi EJ (2018) Seasonal dynamics and potential drivers of ranavirus epidemics in wood frog populations. Oecologia 188:1253–1262

Hallett SL, Ray RA, Hurst CN, Holt RA, Buckles GR, Atkinson SD, Bartholomew JL (2012) Density of the waterborne parasite *Ceratomyxa shasta* and its biological effects on salmon. Appl Environ Microbiol 78:3724–3731

Hartikainen H, Okamura B (2015) Ecology and evolution of malacosporean-bryozoan interaction. In: *Myxozoan Evolution, Ecology and Development*. Okamura B, Gruhl A, Bartholomew JL (eds) Springer International Publishing, Basel, p 201–216

Helps C, Reeves N, Tasker S, Harbour D (2001) Use of real-time quantitative PCR to detect *Chlamydophila felis* infection. J Clin Microbiol 39:2675–2676

Hinlo R, Furlan E, Suitor L, Gleeson D (2017) Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. Manag Biol Invasions 8:89–100

Hunter ME, Dorazio RM, Butterfield JSS, Meigs-Friend G, Nico LG, Ferrante JA (2017) Detection limits of quantitative and digital PCR assays and their influence in presence

– absence surveys of environmental DNA. Mol Ecol Resour 17:221–229

Jane SF, Wilcox TM, Mckelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR (2015) Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Mol Ecol Resour 15:216–227

Julian JT, Glenney GW, Rees C (2019) Evaluating observer bias and seasonal detection rates in amphibian pathogen eDNA collections by citizen scientists. Dis Aquat Org 134:15–24

Klymus KE, Merkes CM, Allison MJ, Jackson CA, Goldberg CS, Helbing CC, Hunter ME, Lance RF, Mangan AM, Monroe EM, Piaggio AJ, Stokdyk JP, Wilson CC, Richter CA (2019) Reporting the limits of detection and quantification for environmental DNA assays. Environ DNA 00:1–12

Lacoursière-Roussel A, Côté G, Leclerc V, Bernatchez L (2016) Quantifying relative fish abundance with eDNA: a promising tool for fisheries management. J Appl Ecol 53:1148–1157

Lacoursière-Roussel A, Deiner K (2019) Environmental DNA is not the tool by itself. J Fish Biol:1–4

Lahoz-Monfort JJ, Guillera-Arroita G, Tingley R (2016) Statistical approaches to account for false-positive errors in environmental DNA samples. Mol Ecol Resour 16:673–685

Lance RF, Guan X (2020) Variation in inhibitor effects on qPCR assays and implications for eDNA surveys. Can J Fish Aquat Sci 77:23–33

Mächler E, Deiner K, Spahn F, Altermatt F (2016) Fishing in the water: effect of sampled water volume on environmental DNA-based detection of macroinvertebrates. Environ Sci Technol 50:305–312

Mächler E, Osathanunkul M, Altermatt F (2018) Shedding light on eDNA: neither natural levels of UV radiation nor the presence of a filter feeder affect eDNA-based detection of aquatic organisms. PLoS One 13:1–15

MacKenzie DI, Nichols JD, Lachman GB, Droege S, Royle AA, Langtimm CA (2002) Estimating site occupancy rates when detection probabilities are less than one. Ecology 83:2248–2255

Maguire C, DiRenzo G V., Tunstall TS, Muletz CR, Zamudio KR, Lips KR (2016) Dead or alive? Viability of chytrid zoospores shed from live amphibian hosts. Dis Aquat Org 119:179–187

Makkonen J, Strand DA, Kokko H, Vrålstad T, Jussila J (2013) Timing and quantifying *Aphanomyces astaci* sporulation from the noble crayfish suffering from the crayfish plague. Vet Microbiol 162:750–755

McClintock BT, Nichols JD, Bailey LL, MacKenzie DI, Kendall WL, Franklin AB (2010) Seeking a second opinion: uncertainty in disease ecology. Ecol Lett 13:659–674

Miaud C, Arnal V, Poulain M, Valentini A, Dejean T (2019) EDNA increases the detectability of ranavirus infection in an alpine amphibian population. Viruses 11:1–15

Minamoto T, Naka T, Moji K, Maruyama A (2016) Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. Limnology 17:23–32

Mosher BA, Bernard RF, Lorch JM, Miller DAW, Richgels KLD, White CLA, Campbell Grant EH (2020) Successful molecular detection studies require clear communication among diverse research partners. Front Ecol Environ 18:43–51

Nichols JD, Bailey LL, O'Connell AF, Talancy NW, Campbell Grant EH, Gilbert AT, Annand EM, Husband TP, Hines JE (2008) Multi-scale occupancy estimation and modelling using multiple detection methods. J Appl Ecol 45:1762–1769

Reeder NMM, Pessier AP, Vredenburg VT (2012) A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. PLoS One 7:1–7

Renshaw MA, Olds BP, Jerde CL, Mcveigh MM, Lodge DM (2015) The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. Mol Ecol Resour 15:168–176

Robinson CV, Uren Webster TM, Cable J, James J, Consuegra S (2018) Simultaneous detection of invasive signal crayfish, endangered white-clawed crayfish and the crayfish plague pathogen using environmental DNA. Biol Conserv 222:241–252

Rocchi S, Tisserant M, Valot B, Laboissière A, Frossard V, Reboux G (2017)

Quantification of *Saprolegnia parasitica* in river water using real-time quantitative PCR: from massive fish mortality to tap drinking water. Int J Environ Health Res 27:1–10

Rusch JC, Hansen H, Strand DA, Markussen T, Hytterød S, Vrålstad T (2018) Catching the fish with the worm: A case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Parasites and Vectors 11:1–12

Rusch JC, Mojžišová M, Strand DA, Svobodová J, Vrålstad T, Petrusek A (2020) Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. NeoBiota 58:1–32

Schmidt BR, Kéry M, Ursenbacher S, Hyman OJ, Collins JP (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. Methods Ecol Evol 4:646–653

Sedlak RH, Kuypers J, Jerome KR (2014) A multiplexed droplet digital PCR assay performs better than qPCR on inhibition prone samples. Diagn Microbiol Infect Dis 80:285–286

Sepulveda AJ, Nelson NM, Jerde CL, Luikart G (2020) Are environmental DNA methods ready for aquatic invasive species management? Trends Ecol Evol 35:668–678

Sepulveda AJ, Schabacker J, Smith S, Al-Chokhachy R, Luikart G, Amish SJ (2019) Improved detection of rare, endangered and invasive trout in using a new large-volume sampling method for eDNA capture. Environ DNA 1:227–237

Shogren AJ, Tank JL, Andruszkiewicz E, Olds B, Mahon AR, Jerde CL, Bolster D (2017) Controls on eDNA movement in streams: transport, retention, and resuspension. Sci Rep 7:5065

Song JW, Small MJ, Casman EA (2017) Making sense of the noise: the effect of hydrology on silver carp eDNA detection in the Chicago area waterway system. Sci Total Environ 605–606:713–720

Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SST, Sigsgaard EE, Hellström M (2017) Comparison of capture and storage methods for aqueous

macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods Ecol Evol 8:635–645

Strand DA, Johnsen SI, Rusch JC, Agersnap S, Larsen WB, Knudsen SW, Møller PR, Vrålstad T (2019) Monitoring a Norwegian freshwater crayfish tragedy: eDNA snapshots of invasion, infection and extinction. J Appl Ecol 56:1661–1673

Strickler KM, Fremier AK, Goldberg CS (2015) Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biol Conserv 183:85–92

Svoboda J, Kozubíková-Balcarová E, Kouba A, Buřič M, Kozák P, Diéguez-Uribeondo J, Petrusek A (2013) Temporal dynamics of spore release of the crayfish plague pathogen from its natural host, American spiny-cheek crayfish (*Orconectes limosus*), evaluated by transmission experiments. Parasitology 140:792–801

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. Vet Microbiol 137:146–155

Wilcox TM, McKelvey KS, Young MK, Sepulveda AJ, Shepard BB, Jane SF, Whiteley AR, Lowe WH, Schwartz MK (2016) Understanding environmental DNA detection probabilities: A case study using a stream-dwelling char *Salvelinus fontinalis*. Biol Conserv 194:209–216

Wittwer C, Nowak C, Strand DA, Vrålstad T, Thines M, Stoll S (2018a) Comparison of two water sampling approaches for eDNA-based crayfish plague detection. Limnologica 70:1–9

Wittwer C, Stoll S, Strand D, Vrålstad T, Nowak C, Thines M (2018b) EDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability. Hydrobiologia 807:87–97

Wood SA, Pochon X, Laroche O, von Ammon U, Adamson J, Zaiko A (2019) A comparison of droplet digital polymerase chain reaction (PCR), quantitative PCR and metabarcoding for species-specific detection in environmental DNA. Mol Ecol Resour 19:1407–1419

Acknowledgements

First of all I would like to thank my advisors Hanna Hartikainen and Christoph Vorburger for giving me the opportunity for this PhD and for lending me their tremendous support for this project. I enjoyed working and discussing my research with you and I always felt to be in good hands with you. I learned a lot from you and I appreciate all the time you have spent helping me.

Secondly, a special thanks goes to Trude Vrålstad for inspiring discussions during the committee meetings, for being the external examiner at my PhD defense, and for allowing me to visit your lab in Oslo. I had a wonderful time collecting water samples in the beautiful Norwegian countryside with Johnny Rusch and David Strand, which I thank as well.

I further would like to thank Armin Zenker and Raphael Krieg for their collaboration on this project, numerous helpful discussions and collecting the major part of the field samples for the country-wide survey. A special thank you goes to Alex King for extracting and analysing a large number of samples and to Anna Weston for her support in the lab.

I am very grateful to Jukka Jokela, who has supported me from the very start as a bachelor student and has offered valuable inputs on various occasions. Thank you for supporting me through all those years. Without you, I probably would not have done my PhD at Eawag. You have a way of inspiring people, me included, to get the most out of their research and to "think big".

A special thank you goes to Raffael Stegmayer, Pravin Ganesanadamoorthy, Pascal Reichlin, Marco Thali, Paula Rodriguez, Marta Reyes, Jana Jucker, Sarah Bratschi, Bettina Dubach, Nora Weissert, Silvana Käser, Tamara Schlegel and Oliver Subotic for their technical support. I would not have been able to complete this project without your contribution in the lab and field. Thank you, also, Gioia Matheson and Arianne Maniglia, you were always ready to help in all kinds of issues, including administrative ones.

I would like to thank all my past and present fellow PhD students and office mates. Especially, but not exclusively, Anja Felmy, Natalia Zajac, Lynn Govaert, Luca Carraro, Robert Dünner, Heidi Käch, Teo Cereghetti, Linda Haltiner, Elvira Mächler, Jeanine Brantschen and Eva Cereghetti. Monday mornings were made more bearable because of you! Also, sharing our ideas, motivations, and, yes, lamenting on how hard life as a PhD can be, made my days at Eawag even more enjoyable.

The ECO department at Eawag is an amazing place to work, with too many people I am thankful of, to name them all. I would therefore want to say many thanks to all former and present ECO members, for many interesting discussions over lunch and more than one fun evening.

Finally, I thank my family for their never-ending support and encouragement, and Renato for believing in me no matter what I do and what will come next.