eDNA-based detection of the crayfish plague, *Aphanomyces astaci* (Schikora 1906), in Germany

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Abstract

Biodiversity is threatened worldwide because of ongoing habitat loss and fragmentation, overexploitation, pollution, biological invasions and a changing global climate. Due to the major importance of biological diversity for modern human living, efficient conservation and management strategies are required to protect endangered habitats and species. For this purpose, ambitious multilateral agreements on regional and global scale were declared to prevent biodiversity loss.

Efficient biomonitoring methods are required to adequately implement these biodiversity conventions. Species monitoring as a core activity in biodiversity research is an effective tool to assess the status of species and trends within habitats. Data collection can be obtained with visual, electronic or genetic surveys. Still, these monitoring programs can be expensive, laborious and inefficient for accurate species assessments. New techniques based on environmental DNA (eDNA) allows for the detection of DNA traces in environmental samples (soil, sediment, water and air samples) and open up new possibilities for species monitoring. The eDNA methodology enables detection of single species in a qualitative (presence/absence) or (semi-) quantitative way. eDNA metabarcoding approaches can be an effective community structure assessment method.

This thesis, located at the interface between experimental and applied research, illustrates the suitability of the eDNA methodology in applied biomonitoring using the example of the water-borne crayfish plague pathogen *Aphanomyces astaci* (Schikora 1906). The obtained results provide new insights into *A. astaci* sporulation dynamics in natural water courses. *A. astaci* sporulation is influenced by seasonal variation of water temperatures and life history traits (molting, activity, mating) of infected crayfish. The results also imply a high transmission risk of *A. astaci* spores during the complete year. This thesis compares two eDNA methods, which are successfully and consistently detecting *A. astaci* spores. Each approach is suitable for different biomonitoring tasks due to the method-specific requirements. The obtained results also reveal spatial variation in *A. astaci* occurance in the tested water bodies. *A. astaci* spore estimates are positively correlated with population density and pathogen loads of captured *A. astaci*- positive crayfish. eDNA results show a downstream zoospore transport of up to three kilometres distance from a distribution hot spot area of *A. astaci*-infected crayfish. The eDNA methodology is helpful in gaining reliable information on *A. astaci* occurrence in large water bodies. This information is urgently needed to initiate efficient management decisions for the conservation of European crayfish species.

eDNA-based methods such as for *A. astaci* detection are a useful complement for conventional monitoring and should have a strong impact on conservation policy. eDNA methodology will be helpful for the practical implementation of the main aims of key conservation agreements and thus will make important contributions to biodiversity protection.

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General Introduction

1 Biodiversity

Biodiversity or biological diversity refers to the variability of all living organisms in terrestrial and aquatic (marine, freshwater) ecosystems and their complex ecological interrelations (CBD 1992). This definition integrates all levels of biological organization including genes, species, ecosystems and functional properties (Wilcox 1984). Genes are the fundamental source of biodiversity at all biological levels (Wilcox 1984). Genetic diversity is essential for a species' reaction on changing environments (Bijlsma and Loeschcke 2012). Genetic variability increases a species' chance to survive, adapt and finally evolve by natural selection (Fisher 1930). Taxonomic diversity describes the total number of taxa or species in a habitat. Ecosystem diversity characterizes the complexity of natural habitat types or ecological systems within an ecosystem. Functional diversity describes the variety of ecological functions and processes realized in an ecosystem (Tilman 2001), e.g. food web interactions (e.g. Bardgett and van der Putten 2014) or ecological services (e.g. de Vries et al. 2013). Changes at one biological level can cause notable effects on other levels due to complex ecological interactions (Wilcox 1984, Hughes et al. 2008).

1.2 Importance of biodiversity

Human well-being is influenced by the beauty of stable ecosystems (Daily et al. 2000) shown by people's connection to their home region through typically occurring species and landscapes. Healthy ecosystems also provide important natural goods and ecosystem services (Díaz et al. 2006). Moreover, diverse ecosystems are highly resistant against disturbances e.g. the spread of non-indigenous intruders (Kennedy et al. 2002). Regions with high biological diversity are associated with larger economic value, as numerous jobs are created in gastronomy and tourism (Vaughan 2000). High biodiversity also increases productivity in forestry (Zhang et al. 2012), agriculture (Kirwan et al. 2007) and fisheries (Dudgeon et al. 2006). Genetic resources or biochemical compounds found in biodiversity hotspots are important drivers for innovation in medicine, pharmacology (David et al. 2015) and biotechnology (Bull et al. 1992).

1.3 Threats to biological diversity

Biological diversity is threatened worldwide due to various human-mediated pressures in all natural ecosystems (Newbold et al. 2015, Costello et al. 2010, Dudgeon et al. 2006, Vorosmarty et al. 2010). The loss of biodiversity is thereby promoted by synergistically acting driving forces (e.g. Bellard et al. 2016, Brook et al. 2008, Mantyka-Pringle et al. 2012, Thomas at al. 2004).

1.3.1 Habitat loss and fragmentation

Habitat loss and deterioration is a consequence of urban expansion, e.g. due to excessive deforestation (e.g. Laurance et al. 2014, Seto et al. 2012) or river regulation (Ward et al. 1999). Extensive habitat change often results in fragmented habitat patches (Hagen et al. 2012). The dispersal potential and gene flow between these fragmented habitats is commonly low (Liao and Reed 2009). Thus small and spatially isolated populations are highly susceptible to random genetic drift (Wright 1931) or inbreeding depression (Keller and Waller 2002). The loss of genetic variability reduces the potential to adapt to changing environmental conditions (Lande and Shannon 1996), which can lower overall fitness and increase sensitivity to environmental stressors (Frankham 2005, Keller and Waller 2002, Lande 1995). Genetically impoverished populations have a higher risk of extinction (Frankham 1995, 2005).

1.3.2 Overexploitation

Natural wildlife populations and natural resources are nowadays utilized in an unsustainable way. This often results in excessive exploitation of wild animal and plant species or their derivatives (e.g. Benítez-López et al. 2017, Ripple et al. 2015, Hutchings 2000, Laurance et al. 2012). Overexploitation of species with high economic and gastronomic value (e.g. Purcell et al. 2014) leads to collapsing populations (Essington et al. 2015, Ripple et al. 2015).

1.3.3 Pollution

Environmental pollutants can be found in all environmental compartments (air e.g. Novák et al. 2009; water e.g. Rivetti et al. 2017, Woodward et al. 2012; soil e.g. Tsiafouli et al. 2015). Organic (e.g. pesticides, Hallmann et al. 2014) and inorganic (e.g. heavy metals, Tyler 1975) compounds originate from urban, industrial and agricultural areas. Depending on the biochemical and ecological pathways, pollutants can have deleterious

effects on various biological levels. This includes fitness loss (e.g. Besseling et al. 2013) or loss of biodiversity (e.g. Tsiafouli et al. 2015).

1.3.4. Biological invasions

The introduction of non-indigenous species and infectious diseases is facilitated by global trade and transport of livestock and wildlife species (Fèvre et al. 2006, Hulme 2009). Invasive alien **s**pecies (IAS) are either introduced unintentionally, e.g. via ballast water (Ruiz et al. 1997), or intentionally, e.g. as biological control (Magnuson et al. 1975) or for stocking purposes (Olden et al. 2006, Rahel 2002). Once released and established in a new environment, invasive species can cause adverse effects on the native biodiversity (Strayer 2010). IAS can induce the collapse of native populations due to higher competitiveness (e.g. Albins and Hixon 2013). The hybridization with closely related species can lead to a replacement of native species (Todesco et al. 2016). Often pathogens are co-introduced with IAS (Fisher et al 2012). These factors contribute to a high extinction risk of native species (Bellard et al. 2016, Clavero and García-Berthou 2005).

1.3.5 Climate change

Global climate change has considerable effects on all biological levels (Parmesan 2006, Walther et al. 2002). Changing climatic conditions can decrease genetic diversity due to directional selection (Bellard et al. 2012). These conditions can also lead to shifts in phenology (timing of life cycle events, e.g. flowering or migration, Root et al. 2003) or distribution and range (Feeley and Silman 2010, Walther et al. 2002). Moreover, climatic changes can influence species interactions (Cahill et al. 2012), the composition and dynamics of communities (Walther et al. 2002) and ecosystem functions (McCarty 2001).

1.4 Protection of biodiversity

Numerous multilateral agreements on the regional and global scale aim to protect the environment (Mitchell 2003). On an international level several key conservation agreements to protect biodiversity are of great importance for European environmental legislation (Table 1).

Table 1 Overview of international key conservation agreements for biodiversity protection.

Convention		Year of implementation	Main aims
Convention on the Conservation of European Wildlife and Natural Habitats	Bern Convention	1979	- Protection of wild animal and plant species and their habitats with special regard to endangered and vulnerable species including migratory species
Convention on International Trade in Endangered Species of Wild Fauna and Flora	CITES	1979	- Safeguard wild animal and plant species (alive, dead or derivatives) from unsustainable exploitation in international wildlife trade - List of endangered and exploited species - Ban or restriction for trade of species threatened with extinction or over-exploitation
Convention on Biological Diversity	CBD	1993	- Conservation and sustainable utilization of components of biological diversity
	Cartagena Protocol	2003	- Regulation of genetically modified organisms
	Nagoya Protocol	2014	- Fair and equitable benefit sharing of using genetic resources (e.g. access to resources, technology transfer, funding)

The CBD Aichi Biodiversity Targets (Leadley et al. 2014) were phrased as strategic plan for biodiversity protection until 2020 and incorporates the following aims:

(1) Protection of biodiversity by reducing the rate of habitat loss and degradation, exploitation of natural resources, pollution and introduction of IAS

(2) Restoration and maintaining the integrity and functioning of biodiversity on all biological levels, also in the face of climate change

(3) Conservation of large and well connected areas of landscapes and seascapes with particular importance for biodiversity and ecosystem services

(4) Promotion of the value and sustainable use of biodiversity in government, society, industry and the agriculture, forestry and fisheries sector.

These targets are incorporated in the EU 2020 Biodiversity Strategy (COM 2011/244 final) and get realized with already implemented EU Directives and regulations (Table 2).

Table 2 Overview of EU Directives and regulations.

Directives and regulations		Year of implementation	Main aims
Directive 79/409/EEC, repealed by 2009/147/EC	Birds Directive	1979	- Protection of all wild birds and their specific habitats on the European continent - Natura2000 sites
Directive 92/43/EEC, amended by 2006/105/EC	Habitats Directive; implementation of the Bern Convention in EU nature law	1992/2006	- Protection of rare and endangered animal and plant species with a special focus on area preservation to reach a 'favorable conservation status' of habitats - Natura2000 sites
Directive 2000/60/EC	Water Framework Directive (WFD)	2000	- Reach good ecological status (biological, hydrological and chemical) in freshwater systems - Reduction of water pollution - Restoration of connectivity of water bodies
Directive 2008/56/EC	Marine Strategy Framework Directive (MSFD)	2008	- Reach good ecological status (biological, hydrological and chemical) in marine systems - Restoration of marine habitats - Conservation of marine species
Council Regulation (EC) No. 338/97	CITES Implementation in EU nature law	1996	- Control of international and internal wildlife trade in EU - List of endangered species largely corresponding to CITES Appendices plus non-CITES-listed, indigenous species
Commission Regulation (EC) No. 865/2006, amended by No. 100/2008	CITES Implementation in EU nature law	2006/2008	- Provision of detailed rules and practical issues e.g. standard model forms for permits or notifications
Regulation (EU) 1143/2014	IAS regulation	2014	- Identification, control, eradication of invasive alien species posing a threat for EU biodiversity - Prioritized invasive alien species of Union concern (Union list)

The Federal Republic of Germany is an EU member state and a part of many international conservation agreements, and therefore obliged to support and properly implement all signed conventions and EU directives. These were incorporated in the Bundesnaturschutzgesetz (BNatSchG; adopted in 2009, amended in 2017). Furthermore, the German National Biodiversity Strategy "Bundesprogramm Biologische Vielfalt" was implemented in the year 2011. This strategy covers a large number of actions to be taken until 2020 to halt the decline in local biodiversity, e.g. the designation of biodiversity hotspot regions with characteristic species and habitats.

1.5 Describing and assessing the diversity of species

Efficient biomonitoring methods are required to adequately implement the above-described legally binding biodiversity conventions. First, one must understand how to describe biodiversity. Levels of diversity can be described in hierarchical scales from alpha to gamma diversity (Whittaker 1960, 1972). Alpha diversity describes the local species diversity within habitats. Beta diversity describes the differentiation between habitats along environmental gradients. Alpha and beta diversity are both determining the total species diversity within a landscape, the gamma diversity. Alpha and gamma diversity can be measured with *species richness*. Species richness describes the number of species per individuals in a particular area, community or ecosystem (Colwell and Coddington 1994). Biological diversity generally comprises the components richness and evenness (Magurran 2004). Thus diversity indices should be used, which consider species richness as well as their relative contribution, e.g. the Simpson index (Simpson 1949) and Shannon-Wiener index (Shannon and Weaver 1963). Beta diversity is measured as species turnover to quantify differentiation among habitats (e.g. Winberg et al. 2007). Recently, occupancy modelling (e.g. Iknayan et al. 2014) is used to estimate species richness and diversity.

Diversity estimates are often used as surrogate for biodiversity (Margules and Pressey 2000). These estimates are based on gathered biomonitoring data. Species monitoring as core activity of biodiversity research (Marsh and Trenham 2008) assesses the condition of species on a regular basis (Kull 2008). Biodiversity assessments evaluate the current status and population trends with the help of attributes. These attributes can be categorized in quantity (e.g. presence/absence, range, population size, density), structure (e.g. genetic diversity, sex ratio) or dynamics (e.g. population decline, recruitment, migration; Hill et al. 2005). The main purpose of monitoring (Fig. 1) is *"to collect information that can be used for development of conservation policy, to examine the outcomes of management actions and to guide management decisions*" (Kull 2008).

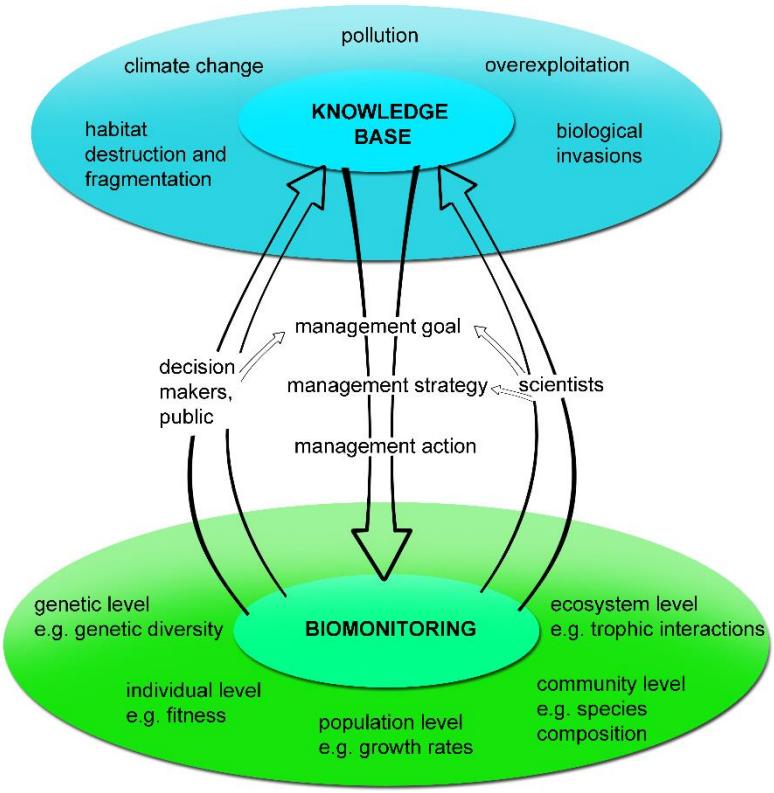

Figure 1 Purpose of biomonitoring. Efficient biomonitoring enables scientists, decision-makers and public stake holders to draw informed conclusions for effective conservation strategies and management actions to protect biodiversity. Information on the condition of species and ecosystems and their reaction to natural or human-induced stressors gathered via biomonitoring increases the knowledge base of all participants. Based on this knowledge, management strategies can be evaluated and adjusted (modified from Niemelä 2000).

Today a wide variety of standardized biomonitoring methods exist for various species and taxa. Visual encounter surveys rely on site visits to survey a species' distribution and abundance (e.g. Dejean et al. 2012, Menegon 2007). In encounter-based assessments species are identified according to species-specific traits, e.g. morphology or acoustics (e.g. Bálint et al. 2018). Quadrants or transects (Hill et al. 2005), both temporarily (e.g. during breeding season) or permanently (e.g. for several years), can be used to estimate population size of target organisms in large areas (e.g. Mattern et al. 2004, Kouakou et al. 2009). Capture methods are based on a species' behavioural-ecological preferences (light traps e.g. McLeod and Costello 2017, pheromone traps e.g. Weinzierl et al. 2005, baits e.g. Southwood and Henderson 2000, artificial refugia e.g. Glorioso and Waddle 2014) or habitat requirements (pitfall traps e.g. Penny et al. 2017; gill-netting e.g. Argent and Kimmel 2005; window traps e. g. Chapman and Kinghorn 1955). Catch return (e.g. *Catch Per Unit Effort* or CPUE, e.g. Zimmerman et al. 2011) or mark-recapture methods

(e.g. Denoël et al. 2018) can be used to survey abundance, density or dispersal patterns of migratory fish, invertebrates, birds or mammals. Wildlife camera traps enable to survey distribution and behaviour (Burton et al. 2015) or population density (e.g. Silver et al. 2004) of elusive species without direct interference.

Electronic fish counters are commonly used in aquatic surveys to assess attributes such as abundance, species composition or travel direction. These counters are either based on electric resistivity, optical devices (e.g. infrared beams) or hydroacoustics (Lucas and Baras 2000). Electrofishing is commonly used to assess fish biodiversity in freshwater systems (e.g. Peterson et al. 2004, Wilcox et al. 2016). Audio surveys can be used for species detection, especially for species-specifically communicating species such as amphibians (e.g. Bálint et al. 2018, Penny et al. 2017, Zimmerman 1994). Radio telemetry is used in aquatic and terrestrial environments to monitor important life style traits such as foraging behavior (e.g. Trivelpiece et al. 1986) or migratory movements (e.g. Bubb et al. 2002).

DNA barcoding and related approaches (i. e. the use of short mitochondrial sequence information for the delineation of species or operational taxa) is often used to identify and discover species (e.g. Coissac et al. 2016, Hebert et al. 2003, Stoeckle 2003), to assess diversity (e.g. Bálint et al. 2012, Leray and Knowlton 2015) or to discriminate cryptic species (e.g. Johnson et al. 2008). Usually tissue samples are collected at survey sites and stored in bottles filled with preserving liquids (e.g. 96% ethanol) for subsequent molecular genetic analysis. The sampling of tissue is invasive, since commonly samples derive from body parts or whole organisms (e.g. larvae, Bálint et al. 2012). Sampling of tissue gets increasingly difficult, the rarer the species is. This is particularly true for many endangered species listed in protection annexes. Sample collection across national borders can be delayed due to strict export/import regulations of the involved countries. To avoid these ethical and regulatory difficulties, researchers can sample rare and endangered species in a non-invasive way with forensic samples. Faeces (e.g. Palomares et al. 2017, Pearson et al. 2014), urine (Sastre et al. 2009), blood spots/oestrus blood (Scandura 2005), bone remains (Germonpré et al. 2009), shed skin (Tawichasri et al. 2017), fur (Frosch et al. 2014, Tsaparis et al. 2014) or molted feathers (Kleven et al. 2016, Rudnick et al. 2005) can be collected from survey site visits. Moreover non-invasive trap solutions exist to collect forensic samples such as hair (Reiners et al. 2011, Steyer et al. 2013). Non-invasive genetic monitoring allows for identification of individuals (e.g.

Rudnick et al. 2005), sexes (e.g. Bidon et al. 2013, Sastre et al. 2009, Tawichasri et al. 2017) or to assess genetic diversity (e.g. Germonpré et al. 2009, Tsaparis et al. 2014) and population size (e.g. Frosch et al. 2014).

Despite the many benefits of both invasive and non-invasive monitoring, these programs are expensive, laborious and network-oriented. Many monitoring projects suffer from inadequate funding, poor study design and deficient implementation. Thus monitoring data obtained from conventional methods can be insufficient and unreliable (Sisk et al. 1994). Moreover a direct link between biomonitoring, applied conservation management and decision-making is needed to implement efficient management strategies. A major challenge for many conventional methods is the high number and variety of species and habitats to be recorded (Creer et al. 2016). These approaches often rely on the monitoring of well-characterized taxa (Bourlat et al. 2013), but only a few species groups can be assessed in an efficient and systematic way. The taxonomic coverage of difficult species groups (e.g. microorganisms or fungi) or species-rich groups in biodiversity hotspots often remains superficial (Bourlat et al. 2013). Additionally, conventional methods can fail to adequately record rare and elusive species (Gu and Swihart 2004). Thus conventional monitoring results often do not or only insufficiently reflect the true species and/or habitat condition. However, recent technological advances in molecular biology support the development of innovative methods for efficient species monitoring. New approaches based on the detection of DNA traces in environmental samples can open up promising opportunities in biodiversity research.

2 Environmental DNA

Environmental DNA (eDNA) is extractable DNA from environmental samples such as soil, sediment, water and air (Taberlet et al. 2012a). Detectable eDNA originates partly from intact or dead cells or cell compounds and from free molecules derived from cellular degradation processes (Barnes and Turner 2016). The eDNA pool is a complex genetic mixture of all organisms, which historically or presently occur in this habitat (Thomsen et al. 2012a, Thomsen and Willerslev 2015).

2.1 Sampling

eDNA can be collected from aerosol samples by filtering air through specific filters and apparatus in a definite time (Bartlett et al. 1997, Folloni et al. 2012, Fig. 2). Soil and sediment-bound eDNA can be sampled on the ground surface with spatula or in deep layers with core samplers (Bienert et al. 2012). eDNA from marine and freshwater systems can be sampled with water column samplers at defined depths (e.g. Adrian-Kalchhauser and Burkhardt-Holm 2016) or from the water surface. Water samples can be collected in small sample tubes (e.g. Ficetola et al. 2008, Thomsen et al. 2012a, Sigsgaard et al. 2015) or with a wide variety of filtration procedures (Table 3).

Table 3 Overview of filtration procedures.

Filter type	**Final water volume**	**References** (studied taxon)
Glass fiber filter	1-10 L	Agersnap et al. 2017 (invertebrate), Jerde et al. 2011 (fish), Olson et al. 2012 (amphibia), Strand et al. 2014 (fungi), Mahon et al. 2013 (fish), Jane et al. 2015 (fish), Fukumoto et al. 2015 (amphibia), Bálint et al. 2018 (amphibia), Sato et al. 2017 (fish)
Cellulose acetate filter	1-2 L	Takahara et al. 2013 (fish), Santas et al. 2013 (amphibia)
Cellulose nitrate filter	1-10 L	Goldberg et al. 2011 (amphibia), 2013 (mollusc), Pilliod et al. 2013, 2014 (amphibia), Laramie et al. 2015 (fish), Shaw et al. 2016 (fish), Spear et al. 2015 (amphibia)
Nylon filter	50- 500 mL	Thomsen et al. 2012b (fish), Bálint et al. 2018 (amphibia)
Polycarbonate filter	2 L	Takahara et al. 2012 (fish)
SterivexTM- GP filter	2 L	Agersnap et al. 2017(invertebrate), Keskin 2014 (fish)
Hollow fibre filter/ tangential flow	10-100 L	Alavandi et al. 2015 (virus), Hill et al. 2005 (microbes)
Hollow fibre filter/ dead-end	100 L	Smith and Hill 2009 (microbes), Strand et al.2014 (fungi)

2.2 Storage

After sampling, environmental samples have to be stored immediately under optimal preserving conditions. eDNA fastly degrades under the influence of microorganisms, endonucleases (Corinaldesi et al. 2008, Zhu 2006) and environmental factors such as high temperature and pH (Strickler et al. 2015). eDNA samples are commonly stored at -20 °C (e.g. Jerde et al. 2011, Thomsen et al. 2012a, Takahara et al. 2012) or in alcohol (e.g. Goldberg et al. 2011, 2013, Ficetola et al. 2008, Pilliod et al. 2013). eDNA can also be preserved in Longmire's buffer (e.g. Renshaw et al. 2015), CTAB buffer (e.g. Balint et al. 2018) or RNAlater (Qiagen; e.g. Spens et al. 2017). Moreover dehydration with silica gels allows for long-term eDNA storage (e.g. Bálint et al. 2018).

2.3 Extraction

The eDNA extraction can be conducted with various procedures. The choice of a suitable eDNA extraction procedure is mainly influenced by the eDNA source or capture material (e.g precipitate or filter), the initial sample volume and the presence of inhibitors. The two-phase-extraction based on phenol-chloroform is a well-tried, low-priced and very effective DNA purification method (e.g. Deiner and Altermatt 2014, Dougherty et al. 2016, Strand et al. 2014). Two-phase-extraction protocols are often specifically developed compositions for specific target organisms and sample types. Column-based extraction methods are mainly commercial purification kits of biotechnology companies such as Qiagen (f. ex. DNeasy Blood and Tissue Kit, e.g. Goldberg et al. 2011; Mobio PowerWater DNA Isolation Kit, e.g. Jane et al. 2015; Mobio PowerWater Sterivex DNA Isolation Kit, e.g. Agersnap et al. 2017).

2.4 Analysis

Commonly PCR-based molecular methods are used to screen environmental samples for the presence of target organisms or taxa. Many eDNA studies focus on specific species and can therefore be described as single-species approaches. Standard PCR can be used for reliable detection of a target species, if DNA sequences in an environmental sample are rather long and abundant (Deiner and Altermatt 2014). Yet, most eDNA molecules in environmental samples are very short (~150 bp) and highly degraded due to continuous eDNA hydrolysis (Deagle et al. 2006). Real-time or quantitative PCR (qPCR) allows for the robust detection of these small fragments in environmental samples. Probe-based qPCR is highly sensitive and reliably detects very low eDNA quantities and small

fragments in eDNA samples (Goldberg et al. 2016). The sensitivity and specificity of real-time PCR assays get further enhanced by choosing appropriate internal hybridization probes (e.g. TaqMan® probes, Vrålstad et al. 2009). For eDNA analysis it is beneficial to use qPCR master mixes specifically designed for samples with co-occurring inhibitory substances (e.g. TaqMan® Environmental Master Mix 2.0, Jane et al. 2015, Thomsen et al. 2012a). The use of real-time PCR technology also allows for multiplexing of several target species in eDNA samples (Tsuji et al. 2018). Microfluidic chips (e.g. for real-time or digital PCR analysis) can be used for eDNA surveillance of target species in aquatic environments (Baker et al. 2018, Doi et al. 2015, Nathan et al. 2014). eDNA methodology can also be used to survey genetic diversity (Deiner et al. 2017, Parsons et al. 2018) or whole communities and ecosystems via eDNA metabarcoding approaches (e.g. Taberlet et al. 2012b, Thomsen et al. 2012b).

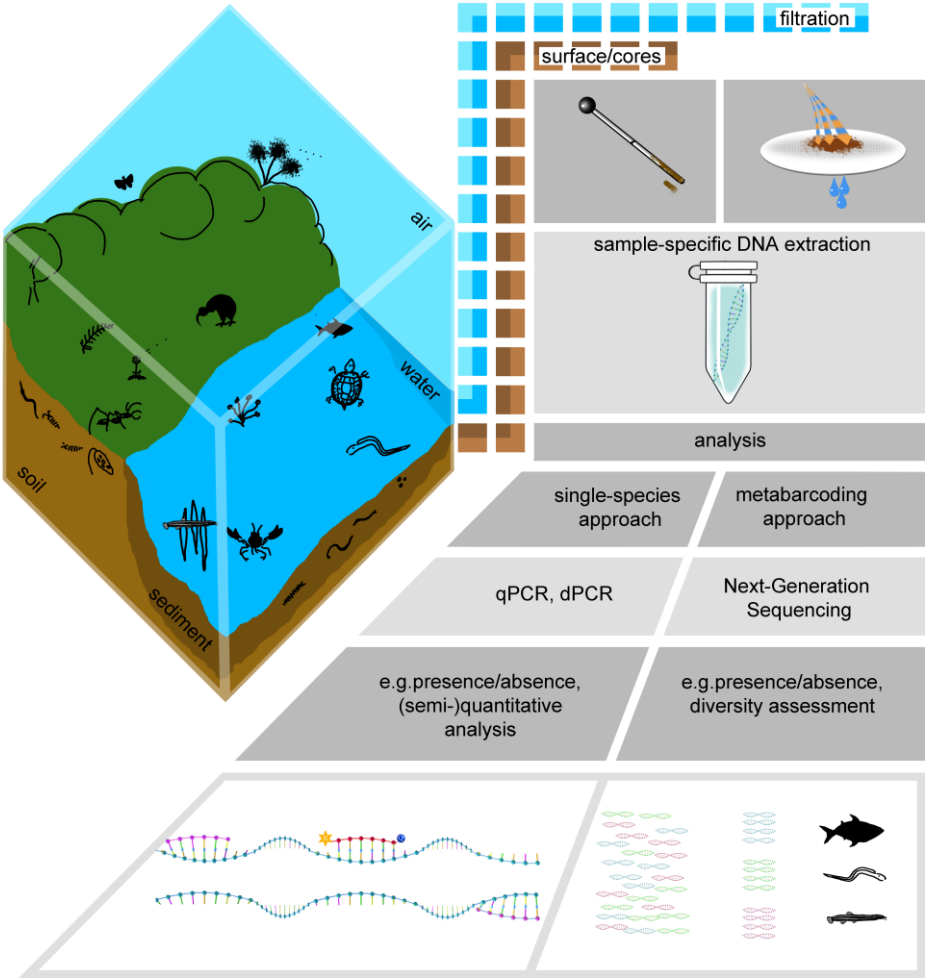

Figure 2 Workflow and basic principles of eDNA methodology. eDNA samples can be collected from different environments with appropriate sampling methods. PCR-based methods allow to screen for single species via qPCR or dPCR (with optional multiplexing) or to use metabarcoding approaches for community assessments.

3 Freshwater crayfish

3.1 Crayfish diversity

Over 640 freshwater crayfish species are described worldwide (Crandall and Buhay 2008). Most species can be found in two biodiversity hotspots located in North America and Australia (Crandall and Buhay 2008). In Europe only five native freshwater crayfish species of the order Decapoda, infraorder Astacidea, superfamily Astacoidea (Hobbs 1988) are present. The noble crayfish *Astacus astacus* (Linnaeus 1758), the stone crayfish *Austropotamobius torrentium* (Paula Schrank 1803) and the white-clawed crayfish *Austropotamobius pallipes* (Lereboullet 1858) occur in Central Europe (Fig.3). The main occurrence of the thick-clawed crayfish *Pontastacus pachypus* (Rathke 1837, WoRMS 2019a) is located in the Ponto-Caspian Basin. The narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz 1823, WoRMS 2019b) occurs in Eastern Europe and the Near East (Souty-Grosset et al. 2006). The central European *A. astacus*, *A. pallipes* and *A. torrentium* are considered as threatened and are included in the Bern Convention, the EU Habitats Directive and IUCN Red list (Holdich et al. 2009, Taugbøl and Skurdal 1999, IUCN 2018).

Freshwater crayfish are the largest invertebrates in freshwater systems (Souty-Grosset et al. 2006). Astacidae are adapted to cold water (Hogger 1988), but the occupied habitats are extremely variable (McMahon 2002). Due to the preferential feeding of detritus in natural habitats, freshwater crayfish positively affect water quality and thus contribute to ecosystem stability (Hogger 1988). They have a key role in ecosystem functioning as consumer of a great variety of food sources (e.g. Guan and Wiles 1998, Saffran and Barton 1993). Crayfish are also important prey for a wide range of predator species (Hogger 1988). The life cycle traits (activity, molting, reproduction) are mainly influenced by water temperature and day length (Hogger 1988, Reynolds 2002).

3.2 Invasive crayfish species

The introduction of non-indigenous invasive crayfish species (NICS) is one of the greatest threats to crayfish biodiversity (Taylor 2002). The European Union's list of invasive alien species (EU Regulation 1143/2014) includes five invasive alien crayfish species: the signal crayfish *Pacifastacus leniusculus* (Dana 1852), spiny-cheek crayfish *Orconectes* (*Faxonius*) *limosus* (Rafinesque 1817, WoRMS 2019c), red swamp crayfish

Procambarus clarkii (Girard 1852), virile crayfish *Orconectes* (*Faxonius*) *virilis* (Hagen 1870; WoRMS 2019d) and marbled crayfish *Procambarus fallax* (Hagen 1870). Invasive crayfish species were introduced to Europe intentionally, e.g. for stocking, or unintentionally, e.g. by pet release (Holdich 1999, Magnuson et al. 1975, Taugbøl and Skurdal 1999). Invasive crayfish species are occupying the same ecological niche as native crayfish species and thus affect their distribution and abundance (Nyström 2002). NICS can replace native crayfish due to a higher competitiveness for food or shelter (Hill and Lodge 1999, Söderbäck 1995). In some cases even mating of closely related species occurs, which results in reproductive interference (e.g. Westman and Savolainen 2001). Invasive crayfish species can also have detrimental effects on the dynamics and diversity of the invaded habitats and communities (Nyström 1999, Holdich 1999) e.g. due to wider food spectrum and increased burrow-building. Major problems arise, if parasites and infectious diseases are introduced together with NICS (Edgerton et al. 2002).

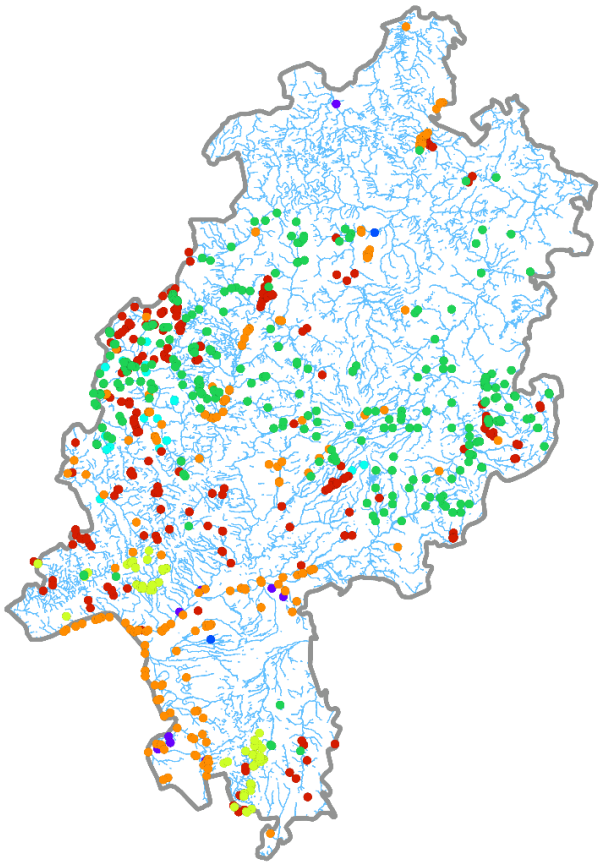

Figure 3 Distribution map of indigenous and non-indigenous crayfish species in the State of Hessen (2017). ● = noble crayfish (*A. astacus*), ● = stone crayfish (*A. torrentium*), ● = narrow-clawed crayfish (*P. leptodactylus*), ● = signal crayfish (*P. leniusculus*), ● = spiny-cheek crayfish (*O. limosus*), ● = red swamp crayfish (*P. clarkii*), ● = marbled crayfish (*P. fallax*). Illustration based on the data provided by the Hessian Agency for Nature Conservation, Environment and Geology, Wiesbaden.

3.3 Crayfish plague (*Aphanomyces astaci*)

The crayfish plague agent *Aphanomyces astaci* (Schikora 1906) is considered to be one of the 100 worst invasive alien species worldwide (Global Invasive Species Database 2018). Recent molecular data classify *A. astaci* in the phylum Chromista, the class Oomycetes, the order Saprolegniales and the family Verrucalvaceae (Beakes et al. 2014). Most likely *A. astaci* was introduced to Europe via importation of infected North American crayfish species (Alderman and Polglase 1988, Unestam 1972). *A. astaci* reached an extensive expansion in Europe mainly due to the ongoing dispersal of invasive alien crayfish species. Confirmed *A. astaci* carriers to date are *P. leniusculus* (Alderman et al. 1990, Unestam 1972), *O. limosus* (Vey et al. 1983), *P. clarkii* (Diéguez-Uribeondo and Söderhäll 1993) and *O. immunis* (Schrimpf et al. 2013). Additionally, other invertebrate species also act as disease vectors (e.g. Schrimpf et al. 2014).

3.3.1 Life cycle and spread

A. astaci is well adapted to aquatic life (Evans and Edgerton 2002) and a highly specialized parasite of crayfish species (Unestam 1969a). The life cycle begins when zoospores locate a suitable host via chemotaxis (Cerenius and Söderhäll 1984a, Fig. 4). Zoospores attach and encyst on a crayfish and discard the flagella (Unestam 1966). The zoospore then germinates and an infection spike penetrates the cuticle. Subsequently, vegetative hyphae branch out in the crayfish's exoskeleton (Nyhlén and Unestam 1975, Unestam and Weiss 1970). The life cycle is completed, when hyphae grow out of the host and build a zoosporangium (Evans and Edgerton 2002). Primary spores are produced and extruded at the sporangium tip. There the spores encyst and form typical "spore balls" (Alderman and Polglase 1986). The infective units, motile biflagellate secondary zoospores, emerge from primary cysts (Alderman and Polglase 1988) and search for new hosts. *A. astaci* spores are continuously released from the infected crayfish (Strand et al. 2012). Zoospore production considerably increases prior to or soon after death (Evans and Edgerton 2002). Secondary zoospores are viable for a few days, but can undergo a process of repeated zoospore emergence (RZE) to find suitable encystment sites (Cerenius and Söderhäll 1984b, Evans and Edgerton 2002). The transmission of *A. astaci* spores generally occurs downstream by water flow or upstream by migrating infected carrier crayfish species. Further spread is also facilitated by contaminated fishing equipment (Alderman et al. 1996) or predators such as otters, birds and eels (Evans and Edgerton 2002).

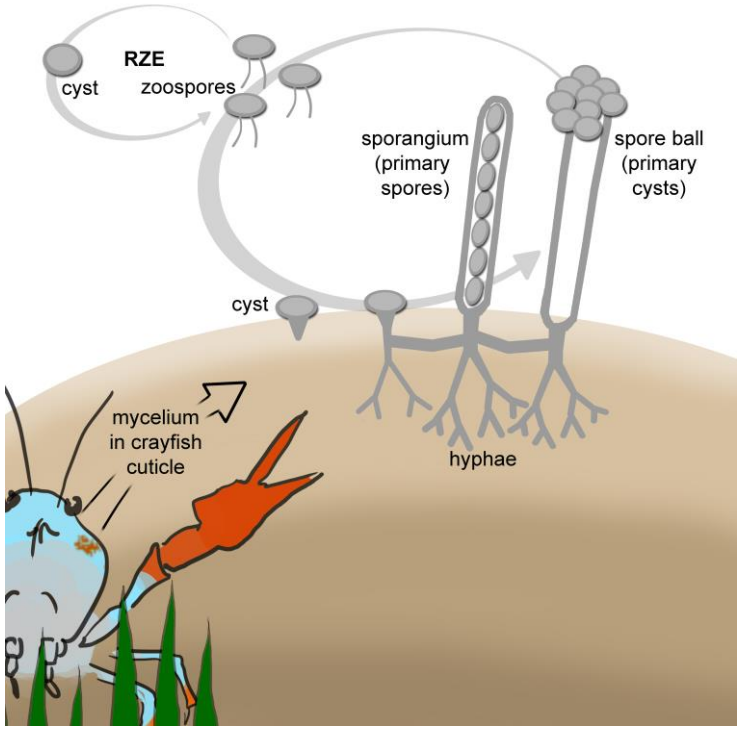

Figure 4 Life cycle of *A. astaci*. Zoospores locate a suitable host and encyst on the crayfish cuticle. The zoospore germinates and vegetative hyphae branch out in the exoskeleton. In the further course of disease hyphae grow out of the host and build zoosporangia with primary spores.These spores are extruded, develop to primary cysts and build "spore balls". From these cysts the infectious secondary zoospores emerge and search for new hosts. Zoospores can undergo a process of repeated zoospore emergence (RZE).

3.3.2 Pathogenicity

To date, five different *A. astaci* genotypes are known (Svoboda et al. 2017). These variants exhibit differing virulences and symptoms in susceptible species (Becking et al. 2015, Jussila et al. 2013, Makkonen et al. 2012, 2014). North American carrier crayfish species are at least partially resistant to *A. astaci* infection, since they developed an efficient immune defence system (Alderman and Polglase 1988). The system controls hyphal growth through melanization (Unestam and Nylund 1972, Unestam and Weiss 1970). Only under stressful conditions (e.g. infections, injuries) normally unsusceptible species can die from *A. astaci* infection (e.g. Persson et al. 1987, Söderhäll and Cerenius 1992). The immune system of European crayfish species largely fails to contain the *A. astaci* infection (Unestam and Weiss 1970). Fast hyphal growth into the body cavity results in the host's death after approximately two weeks (Evans and Edgerton 2002). A high *A. astaci* spore challenge in combination with high water temperature results in short incubation time and rapid mortality in susceptible crayfish (Alderman et al. 1987, Alderman and Polglase 1988).

Thesis objective and aims

In both theory and experiments, eDNA methodology proved suitable for species monitoring. Still, the practical implementation of eDNA methods in applied biomonitoring and conservation management remains stagnant at a low level. The main objective of the thesis is to apply an already established and robust *A. astaci*-eDNA detection system for practical use in biomonitoring assessments and risk management. In order to achieve this objective, the following aims are addressed:

1. Extending the knowledge of spatial and seasonal variation of *A. astaci* sporulation dynamics and detectability in natural water systems (**Publication I, II** and **III**)
2. Providing comparative data of two eDNA methods for the selection of the most promising water filtering procedure for *A. astaci* detection (**Publication II**)
3. Testing the effect of varying population densities and/or pathogen loads of infected crayfish on *A. astaci* detectability with eDNA in natural water systems (**Publication III**)
4. Exploring the potential of eDNA-based *A. astaci* detection as supplement for conventional biomonitoring assessments (**Publication I** and **III**)

Publication I aims to test the effect of seasonally varying water temperatures on *A. astaci* sporulation dynamics in natural water systems. Furthermore, it aims to extend the knowledge of the link between *A. astaci* sporulation and crayfish ecology. This publication also addresses the transmission risk of *A. astaci* spores in the cold season. To achieve this, a year-round simultaneous eDNA monitoring and conventional trap assessment was conducted. **Publication II** aims to compare the suitability of two eDNA techniques for *A. astaci* detection for large-scale biomonitoring assessments. It also addresses the effect of water turbidity on *A. astaci* detection via eDNA. **Publication III** aims to evaluate the use of eDNA-based *A. astaci* detection as a practical tool for biomonitoring and risk management. To achieve this, eDNA and tissue samples of water bodies with varying population densities and/or pathogen loads of infected crayfish were tested for *A. astaci* occurrence. It also aims to reveal local patterns, distribution limits and transport distances of *A. astaci* in natural water bodies.

In the following general discussion, the results and challenges of eDNA and conventional monitoring methods are discussed and placed in the context of current literature. Finally, an outlook and recommendations for the use of eDNA surveys in biodiversity research is provided.

General Discussion

1 eDNA-based species detection

1.1 Historical and recent development

Although not classified as eDNA approaches, microbiologists were the first to investigate environmental samples to detect nucleic acids of hazardous microorganisms. The development from culture-based towards DNA-based approaches was facilitated by the awareness that some microbes are insufficiently or impossible to cultivate (Pace et al. 1986, Giovannoni et al. 1990). The term ‘eDNA’ was first described by Ogram et al. (1987), when extracellular microbial DNA molecules were detected in marine sediment samples. The number of eDNA studies to assess microbial diversity increased considerably over the years. This development widened the scope from waste water and contaminated soils to permafrost (e.g. Steven et al. 2007), pack ice (Brinkmeyer et al. 2003), marine (e.g. Sogin et al. 2006, Venter et al. 2004) and freshwater (e.g. Medinger et al. 2010) samples. Ficetola et al. (2008) were the first to show that macrobial DNA can be detected in freshwater samples. This breakthrough led to an exponential increase in eDNA studies in the 2010s for the detection of various species and taxa (Thomsen and Willerslev 2015).

1.2 Standard diagnostics for *A. astaci* confirmation

The above-mentioned development changed the way of detecting pathogens such as *A. astaci* in aquatic environments. Usually *A. astaci* outbreaks are only suspected when mass mortality of susceptible crayfish species is observed in natural environments (OIE 2018). First clinical signs comprise behavioral abnormalities such as activity during daylight instead of typical nocturnal behaviour, impaired coordination or paralysis and visible external injuries (Alderman et al. 1987). Standard diagnostics to confirm *A. astaci* infection can rely on microscopy (hyphal growth e.g. Nyhlén and Unestam 1975; melanized spots e.g. Unestam and Nylund 1972) or cultivation methods (e.g. Alderman and Polglase 1986; Nyhlén and Unestam 1980). Today, molecular methods based on PCR and sequencing (e.g. Oidtmann et al. 2006) or qPCR (Vrålstad et al. 2009) are highly recommended for *A. astaci* confirmation. These molecular techniques provide higher diagnostic sensitivity, specificity and accuracy compared to the other diagnostic tools (OIE 2018). The downside of all standard diagnostics is their dependence on sampling a sufficiently large number of crayfish tissue. OIE guidelines recommend the use of 5-10

crayfish samples for reliable *A. astaci* confirmation. This sample number should account for variations in *A. astaci* detectability due to varying sample quality and detectable infection sites (OIE 2018). The total number of tissue samples should be increased, if *A. astaci* prevalence of a tested crayfish population is low.

1.3 *A. astaci* ecology and sporulation dynamics

A. astaci is one of the most intensively studied invertebrate pathogen (Alderman and Polglase 1988). Still, little is known about *A. astaci* ecology, its sustainability under natural conditions and factors influencing *A. astaci* sporulation dynamics in the wild. The real-time PCR assay of Vrålstad et al. (2009) allows for the detection of *A. astaci* spores in water samples (Strand et al. 2011, 2012). This facilitated the examination of sporulation dynamics in laboratory experiments. First results showed that *A. astaci* spore release of life infected crayfish is temperature-dependent and occurs continuously (Strand et al. 2012). Spore release was shown to be upregulated during molting (Svoboda et al. 2013) as well as in moribund and recently deceased infected crayfish (Makkonen et al. 2013, Strand et al. 2012). These sporulation patterns led to the suggestion that no time periods can be proclaimed safe of *A. astaci* transmission (Svoboda et al. 2013). The varying capture success of *A. astaci* spores appeared to be influenced by a spatial and temporal heterogeneity of spore distribution (Strand et al. 2011). This difference was thought to be the result of a patchy distribution of crayfish or a low *A. astaci* prevalence in the population. Strand et al. (2014) were the first to show that *A. astaci* spores can be captured via eDNA in large natural water systems. This publication also provided the first evidence of temporal variation of *A. astaci* sporulation dynamics in natural water bodies. **Publication I** revealed that *A. astaci* sporulation dynamics in natural water systems are influenced by seasonal variation of water temperatures and life history traits (molting, activity, mating) of infected crayfish. The full-year assessment led to conclusive evidence that *A. astaci* spores are released from infected crayfish in seasonally varying concentrations. Overall, a high transmission risk of *A. astaci* spores is apparent during the complete year. **Publication III** gives new valuable insights into spatial variation of *A. astaci* occurrence in different water bodies. Obtained results reveal that *A. astaci* spore estimates were positively correlated with population density of infected crayfish populations. Due to this correlation, eDNA sampling enables estimation of the distribution zones as well as distribution limits of *A. astaci*-infected crayfish. Furthermore, individual variation in pathogen load on single crayfish was confirmed to

affect the spore content under natural conditions as shown in previous studies (Strand et al. 2014, Makkonen et al. 2013).

The effective conservation of European crayfish species depends on reliable information on *A. astaci* occurrence. Moreover, profound knowledge on temporal, spatial and individual variations of *A. astaci* sporulation dynamics can be helpful for risk management and can help guide efficient management decisions.

Today eDNA-based monitoring allows confirmation of *A. astaci* occurrence in acute outbreak events (Strand et al. 2014) and chronically infected crayfish populations (**Publications I** and **III**). eDNA monitoring can also detect *A. astaci* at early stages of invasion (**Publication III**). In the future, large-scale eDNA surveillance of natural water bodies could be implemented for early warning of *A. astaci* occurrence. In combination with the analysis of mitochondrial DNA (mtDNA) markers (Makkonen et al. 2018) or mitogenomes (Deiner et al. 2017), *A. astaci* strains causing outbreaks could be characterized. eDNA can be helpful to verify habitats as "*A. astaci*-free" or to screen adjacent water bodies for low- and high-risk carrier populations (Strand et al. 2013) prior to restocking attempts of native crayfish species.

eDNA-based monitoring can also be used as diagnostic tool to control further spread of *A. astaci*. The application of eDNA methodology allows monitoring of zoospore reduction after acute outbreak events or after eradication attempts. The zoospore travel in and between water bodies could be monitored to prevent or forecast new outbreaks. Moreover the monitoring of inlet and outlet water in aquaculture systems cultivating NICS is possible (**Publication III**). eDNA results can be used as basis for verification as "*A. astaci*-free" (Schrimpf et al. 2013) and to distribute health certificates for inspected facilities (Strand et al. 2013).

2 eDNA vs. conventional monitoring

Reliable monitoring data are required for effective conservation strategies and management decisions to protect endangered species (Kull 2008). Here different aspects of conventional and eDNA monitoring are compared with a focus on method-specific similarities, advantages and shortfalls.

2.1 Sampling effort

The effective application of conventional and eDNA-based methods requires expert knowledge of a species' lifestyle and habitat requirements (Herder et al. 2013). Both survey procedures are affected by varying species distribution patterns, which increase sampling effort (Magurran 2004). Moreover both monitoring types exhibit varying ability to detect species as not all species are equally easy to sample (Southwood and Henderson 2000).

Conventional monitoring methods are able to distinguish live animals as well as different life stages (e.g. eggs or larvae, Rees et al. 2014). However these approaches need experienced field workers involving high workloads and physical time in the field (Biggs et al. 2015) to install and maintain adequate monitoring systems. In addition, taxonomic professionals are needed for accurate species identification (Bourlat et al. 2013). Despite these drawbacks, the main advantage of conventional sampling is the possibility to identify errors in sampling scheme or arrangement, which can be corrected by adjusting the sampling process (e.g. with better training, Coddington et al. 1991). Detection probabilities with conventional sampling rise considerably with increased sampling effort (e.g. person-hours, Gu and Swihart 2004).

In comparison to traditional monitoring methods, eDNA-based methods have unique challenges and advantages. During project launches, eDNA-based methods must be properly implemented in terms of sampling, extraction procedures and data analysis, which can be time-consuming and expensive. However, once established and validated, the collection and analysis of eDNA samples is easy, fast and widely applicable (Bohmann et al. 2014). In many studies, eDNA methods were shown to be highly sensitive and exhibited low sampling effort (e.g. Dejean et al. 2012, Smart et al. 2015; **Publication I** and **III**). eDNA-based methods are less labour intensive with less person-hours in the field (Olson et al. 2012). Additionally, the field component of eDNA surveys can be carried out by interested citizens (Biggs et al. 2015, Miralles et al. 2016).

2.2 Coverage of biodiversity levels

Conventional monitoring methods have a long history as standard monitoring techniques in biodiversity research. Genetic diversity is usually determined with tissue (e.g. Bálint et al. 2012) or forensic samples (e.g. Tsaparis et al. 2014). The genetic structure of a population is assessed with marker systems targeting single-nucleotide polymorphisms (SNPs; e.g. von Thaden et al. 2017) or mitochondrial microsatellites (e.g. Steyer et al. 2013). Species monitoring is mainly assessed with the described conventional survey methods due to their effective and well-practiced sampling schemes. Still, the assessment of full species inventories to estimate species diversity is often difficult. The main challenges are the extreme volume and the wide spectrum of species to sample as well as the lack of financial resources (Creer et al. 2016). Conventional methods also often fail to estimate the diversity at higher biodiversity levels (ecosystem diversity, functional diversity and total biodiversity, Sisk et al. 1994). The main reason for this is their focus on a limited number of selected species (e.g. based on 'vulnerable' status, high abundance and/or recognition value; Bourlat et al. 2013). These estimates often do not incorporate temporal and spatial variations. Thus, the complexity of all ecosystems and ecosystem functions is depicted only inaccurately.

The main application of eDNA nowadays is the monitoring of single target species (e.g. Thomsen et al. 2012a). These approaches also allow estimation of a species' abundance or biomass by measuring eDNA concentrations (e.g. Pilliod et al. 2013, Takahara et al. 2012). The estimates give valuable information about a species' ability to raise offspring or to persist in natural habitats (Bohmann et al. 2014). Recent advances in eDNA technology enable genetic assessment of species diversity via water samples (Elbrecht et al. 2018, Parsons et al. 2018). eDNA technology increases the number of monitored species and habitats with the use of Multiplex qPCR assays (Tsuji et al. 2018) or digitalPCR platforms (e.g. Baker et al. 2018). A more 'holistic' view on biodiversity gets realized by using eDNA metabarcoding approaches. eDNA metabarcoding is already used for species detection and community structure assessments for a wide variety of taxa and habitats (e.g. Sato et al. 2017, Shaw et al. 2016, Valentini et al. 2016, Bálint et al. 2018, Port et al. 2016). The application of eDNA metabarcoding also enables estimation of species richness (Deiner et al. 2017, Olds et al. 2016, Valentini et al. 2016) and assessment of spatial (Civade et al. 2016, Nakagawa et al. 2018) and seasonal variation in species composition (Sigsgaard et al. 2017, Stoeckle et al. 2017). Moreover trophic

interactions can be assessed with metabarcoding approaches (Valentini et al. 2009, Pompanon et al. 2012). One of the main challenges of eDNA metabarcoding approaches is the enormous amount of sequencing data, which requires bioinformatic expertise for interpretation. Another problem can be the selection and validation of short, but taxon-specific markers to reach a sufficient taxonomic coverage and resolution with degraded eDNA samples (Valentini et al. 2016, Taberlet et al. 2012b).

2.3 Invasiveness

eDNA methods are generally non-invasive approaches. The possibility to sample water or soil reduces the interference of target organisms in their habitats (Olson et al. 2012, Takahara et al. 2013). Conventional methods vary in their invasiveness depending on the assessment type. For example, the use of camera or audio records or forensic material is non-invasive. These surveys do not interfere with target organisms in natural habitats. In contrast, methods such as electrofishing are invasive. Although this procedure is not harmful, if performed correctly, it can cause injuries or even fatalities (Snyder 2003). Some even more invasive techniques are divers capture methods due to direct interference with the subject species. The application of these methods can result in discomfort, fitness loss or in extreme cases increased mortality (Putman 1995).

2.4 Data consistency: spatial, temporal and taxonomic coverage

Data obtained via conventional biomonitoring methods can be biased due to varying spatial, taxonomic and temporal coverage (Bourlat et al. 2013, Dudgeon et al. 2006). These methods are usually confined to specific, well-known areas and/or times with a high likelihood of a species' occurrence (Hill et al. 2005). Poor consistency in spatial coverage is often the result of an unequal distribution of survey sites (e.g. developed versus developing countries, Sisk et al. 1994) and their accessibility (e.g. remoteness). The coverage of taxonomic data collection is often biased towards species, which are easy to monitor (Bourlat et al. 2013). The temporal coverage (i.e. the record of seasonal changes in species composition and abundance) remains deficient due to the difficulty to repeat conventional surveys.

In comparison, the consistency of eDNA data is mainly influenced by the used capture, storage and extraction procedures (e.g. Deiner et al. 2015, Spens et al. 2017, **Publication II**). Moreover the applied detection system (e.g. qPCR vs. dPCR, Doi et al. 2015) as well as the subsequent data analysis workflow (e.g. Agersnap et al. 2017) can have a strong

influence on data consistency. Other factors that may cause inconsistencies are varying detection success (e.g. very low population densities), and varying biotic and abiotic environmental conditions across ecosystems (e.g. Barnes and Turner 2016, Pietramellara et al. 2009). In comparison to conventional surveys, eDNA methods allow for repeated spatial or temporal measurements (Bohmann et al. 2014, **Publication I-III**). eDNA surveys are not confined to specific habitat fragments and can be applied in all habitats on earth. The taxonomic coverage is far-reaching due to the possibility to design species- or taxon-specific eDNA markers based on available sequencing data.

2.5 Factors influencing detectability

Conventional monitoring methods can fail to detect target species due to method-specific shortfalls. These include the species-specificity of the used method, unfavorable weather conditions (Magurran 2004) or the varying efficiency and expertise of fieldworkers (Coddington et al. 1991). Due to these constraints, conventional monitoring methods can overlook a species' presence or underestimate its abundance. Non-detection errors are especially problematic for rare and elusive species as well as for invasive species in early invasion stages. An uneven distribution in a study area can make it more difficult to sample target species (Gu and Swihart 2004).

The detectability of eDNA varies across ecosystems. Soils and sediments exhibit varying eDNA binding properties (Pietramellara et al. 2009). Anoxic conditions in sediments reduce the eDNA degradation via nucleases (Corinaldesi et al. 2011). Under these conditions, eDNA has the potential to be preserved over long timescales (overview in Thomsen and Willerslev 2015, Willerslev et al. 2003). In water eDNA degrades fast within days to weeks (e.g. Barnes et al. 2014, Dejean et al. 2011, Thomsen et al. 2012a), indicating a contemporary presence of live target species (Sisgaard et al. 2015). eDNA detection probability in aquatic environments was shown to be affected by various factors. These include ecological factors such as seasonally varying activity patterns (Goldberg et al. 2011, de Souza et al. 2016) or the age structure of a population (Maruyama et al. 2014). Moreover diet and biomass was found to affect eDNA concentrations (Klymus et al. 2015). Varying shedding rates of eDNA material (e.g. Deiner and Altermatt 2014, Klymus et al. 2015) can substantially influence the detection success of eDNA in water samples. eDNA persistence and thus detectability is affected by temporally and spatially varying environmental factors such as temperature, pH value, UV radiation or salinity (Barnes and Turner 2016, Strickler et al. 2015). In aquatic

environments eDNA detection probability is also influenced by dilution, distributional patterns (e.g. evenness, depth) and transport (Goldberg et al. 2016). Aquatic sediments hold higher eDNA amounts than surface water (Turner et al. 2015). Moreover the eDNA detectability is higher in stagnant waters compared to running waters (Thomsen et al. 2012a). In lotic systems the eDNA detection success seems to be dependent on the water flow, stream-specific turbulences and transport distances (Jane et al. 2015).

eDNA approaches detect target species without direct visual contact during the complete sampling process. This leads to uncertainties in regard to the reliability and repeatability of eDNA results (Bohmann et al. 2014). Particularly the occurrence and discovery of false positive and false negative results is subject of ongoing research (Darling and Mahon 2011). A false positive error improperly indicates the presence of eDNA in a sample, although the target species or taxon is not present at the sampling site. False negative errors improperly indicate the absence of eDNA in a sample, although the target species or taxon is present in a habitat. These errors can arise from inappropriate sampling and sample handling, deficient eDNA extraction and amplification procedures or stochastic ecological factors (Table 4).

Table 4 Possible sources of false positive and false negative errors.

Error source	False positive	False negative
Sampling	- contamination of sampling equipment and/or materials	- insufficient sampling strategy (e.g. filter type, low water volume, inadequate number of subsamples, disregard of species ecology)
Sample handling	- cross-contamination during transport or lab work	- eDNA degradation due to inadequate sample storage and handling
Extraction and amplification strategies	- insufficient specificity	- insufficient sensitivity and/or specificity - PCR and sequencing errors (NGS) - coextraction of PCR inhibitors (Jane et al. 2015)
Ecological factors	- co-existence of closely related species in one habitat - random eDNA transport mechanisms from one location to another, e.g. • excretion by species preying on target species (Merkes et al 2014) • human-mediated eDNA dissemination by boats or fishing gear (Andersen et al. 2014) • long-distance transport in ballast water tanks (Ruiz et al. 1997) • dissolved eDNA fragments bound to sediments (Roussel et al. 2015)	- uneven distribution of species occurrence - low population densities of target organisms - suboptimal environmental conditions (e.g. high water turbidity) - suboptimal habitat characteristics (e.g. many shelters)

3 Conclusions and recommendations for management and research

3.1 eDNA as complement to conventional monitoring

One of the main aims of recent work in eDNA studies is their inclusion as standard monitoring tools in biodiversity research. eDNA monitoring is not thought to replace, but rather to complement conventional monitoring procedures (Pikitch 2018, **Publication I** and **III**). This could be achieved by implementing two-tiered monitoring programs (Kelly et al. 2014) that combine initial large-scale eDNA screening with subsequent site-specific conventional assessments (e.g. Foote et al. 2012, Wilson et al. 2014). The use of eDNA monitoring is beneficial, especially when conventional biomonitoring surveys are not easy to perform (Creer et al. 2016), labor intensive, or expensive (Kelly et al. 2014). Conventional methods can be ineffective to record all species occurring in a large area (Magurran 2004). Thus full species inventories to assess of alpha and beta diversity are difficult to achieve (Colwell and Coddington 1994, Creer et al. 2016). Moreover the application of conventional methods can be highly destructive for habitats and species (Kelly et al. 2014). Despite these drawbacks, conventional methods cover biodiversity attributes currently undiscernible with eDNA methodology, e.g. age structure or growth rates (Pikitch 2018). Therefore, eDNA methodology is a suitable complement as it is non-invasive and requires less person-hours in the field (Biggs et al. 2015, Sigsgaard et al. 2015). eDNA monitoring does not depend on expert taxonomic knowledge (Pikitch 2018) and can reduce costs for equipment and financial resources (Adrian-Kalchhauser and Burkhardt-Holm 2016). Due to higher sensitivity (e.g. Jerde et al. 2011, Smart et al. 2015, Dejean et al. 2012, **Publication I**), the application of eDNA methods is particularly well suited for rare and elusive species or invasive species. These groups are generally hard to detect with conventional monitoring (Adrian-Kalchhauser and Burkhardt-Holm 2016, Ficetola et al. 2008, Rees et al. 2014).

3.2 Preliminary considerations for eDNA study design

The selection of an appropriate eDNA biomonitoring method depends on various factors, e.g. the research question, the project budget or the number of target species (Creer et al. 2016). Prior to sampling, a species' lifestyle and habitat requirements (Herder et al. 2013) have to be evaluated for adequate study design and interpretation. eDNA detection probabilities for initial testing distinctly increase when historical records and/or recent fishing surveys are considered (e.g. Sigsgaard et al. 2015). A robust eDNA study should

include an adequate number of sampling sites and replicates (technical and biological) for statistical power (Creer et al. 2016). Additionally, data of physical and chemical environmental parameters such as temperature or pH value should be incorporated in the study. The sampling design should take into account temporal variations (e.g. season), water types and varying spatial distribution in habitats (e.g. in certain water depths, Herder et al. 2013). The complete sampling and extraction procedure has to be thoroughly tested to evaluate its applicability for the target species and the surveyed ecoregion. If species occur in low abundances, sampling effort should be increased by more intensive sampling (Gu and Swihart 2004, **Publication II**). Blank samples with sterile water as well as positive (presence) and negative control samples (absence) are used for quality control in the field (Ficetola et al. 2008). eDNA studies are generally conducted in non-invasive laboratories with separate rooms for eDNA extraction and amplification. Strict working and decontamination protocols (e.g. thoroughly cleaned equipment, DNA-free consumables) should be considered to minimize the contamination risk (Olson et al. 2012, Darling and Mahon 2011). More detailed guidelines for eDNA project design and planning, in silico and in vitro testing of primers, chemistries and PCR conditions are provided by Goldberg et al. (2016), Shepherd et al. (2014) and Taberlet et al. (2018).

3.3 Ongoing advances in eDNA technology

Although utilized at an increasing rate in ecology and wildlife monitoring, eDNA technology is still thoroughly tested for the use in large-scale biodiversity assessments. Many open questions exist regarding the best way to accurately sample, extract and analyze eDNA (Bohmann et al. 2014) and are major topics of ongoing basic research. Further improvements in qPCR and NGS technology will lead to increased sensitivity, lower error rates and high sample throughput (e.g. Creer et al. 2016, Ekblom and Galindo 2011).

Recent optimizations lead to a decreasing size of sampling equipment. Mobile eDNA samplers and analysis platforms are currently developed and thoroughly tested for the use in biodiversity assessments. As a result of joint development work of Smith Root and Biomeme the ANDe™ water sampling system coupled with a handheld Biomeme two3™ qPCR device was invented. This system is able to collect and process eDNA from water samples directly in the field (Thomas et al. 2018). The MinION™ (Oxford Nanopore Technologies) is a portable nanopore-based sequencing apparatus. This device is able to sequence environmental specimens directly on site in a fast and cost effective way

(Bayley 2015, Srivathsan et al. 2018). The proof of concept of the MinION™ was demonstrated with model bacterial communities in earlier studies (Benítez-Páez et al. 2016, Mitsuhashi et al. 2017). This device also showed a great promise as a tool for environmental metagenomic analysis (Brown et al. 2017). It was already successfully used in field-based assessments for the identification of animal (Menegon et al. 2017) and plant specimens (Parker et al. 2017). The MinION™ also allows to characterize microbioms (Shin et al. 2016) and bacterial communities in complex environmental samples (Kerkhof et al. 2017). Rapid biodiversity assessments are even possible in extreme and inhospitable environments such as arctic permafrost (Goordial et al. 2017) or remote regions such as tropical forests (Menegon et al. 2017, Pomerantz et al. 2018). Moreover space agencies use nanopore-based biosensors to monitor crew health and to detect extraterrestrial life in space exploration (Castro-Wallace et al. 2017). Further advances in nanopore technology will improve sequence output, decrease error rates (Brown et al. 2017) and enhance sample preparation (Bayley 2015). These advances will lead to a broad application of nanopore-based biosensors in biodiversity assessments.

All progress points to a hand-held detection device comparable to the 'Tricorder' as introduced by the Science Fiction series Star Trek®. This multifunctional device is able to scan the environment, to analyse the data in real-time and to record all incoming information. Recent innovations in the area of micro-/nanofluidics and microfabrication already allow for the analysis of bio/-chemical compounds on small devices. Chemical analysis can possibly be performed on hand-held mass spectrometers (e.g. Blain et al. 2004, Syms and Wright 2016). The invention of lab-on-a-chip devices will enable to diagnose diseases (Foudeh et al. 2012) or to monitor the environment (Pol et al. 2017). With further adjustments, it is quite likely that eDNA molecules can be detected with hand-held "eDNA Tricorders".

3.4 Using eDNA methods in applied biomonitoring and conservation

The loss of biological diversity worldwide due to various human-mediated threats requires rapid and efficient conservation and management strategies. Governmental agencies and private stakeholders require extensive information on the status of species, before protection measures are taken (Sisk et al. 1994). As a complement to conventional biomonitoring methods, the application of eDNA technology can be helpful to guide management decisions (Darling and Mahon 2011, **Publication III**). However, the transfer from experimental to practical utilization of eDNA proves challenging. From

experience in applied eDNA monitoring, one main question is why eDNA surveys are not ordered by public and/or private stakeholders on a regular basis. Today many species- or taxon-specific eDNA assays exist and the number increases steadily. There is high interest in this technique for biomonitoring of rare or invasive species. Furthermore, the number of companies providing eDNA services continually increases in Europe. With this high interest and availability of resources, there are still obstacles preventing interested stakeholders from inquiring and performing routine, large-scale eDNA monitoring. The main reasons for this seem to be that eDNA ecology (i.e. its origin, state, fate and transport; Barnes and Turner 2016) and eDNA workflows are still topics of ongoing basic research. For eDNA methodology to be used as a standard monitoring approach, the data must be reliable and robust. To achieve this goal, problems associated with abundance estimates, detection limits and efficiencies or true false positive and false negative detections must be solved (Darling and Mahon 2011, Ficetola et al. 2015, 2016, Roussel et al. 2015).

Outside of these main issues relating to better methodologies, there is a lack of comprehensive service provision. Often contracting authorities or clients need more detailed information on eDNA results (e.g. detection limits, the effect of environmental factors) to draw informed management decisions (Jane et al. 2015). Thus providers of eDNA surveys should be encouraged to provide comprehensive consultation and communication prior to eDNA sampling and for data interpretation. Particular consideration is needed to interpret conflicting results achieved via eDNA and conventional methods. Moreover, unclear results relating to assay-specific detection limits need to be considered carefully. This comprehensive service also boosts prices for eDNA monitoring. These costs could be reduced by establishing competence centres for eDNA analytics. Interdisciplinary working groups in these centres could explore eDNA ecology and develop standardized eDNA protocols from sampling to final analysis.

Conservation authorities and managers can already draw informed conclusions for conservation management via eDNA-based presence/absence surveys. Large-scale eDNA monitoring programs were already conducted for the detection of protected (Biggs et al. 2015) and alien invasive species (Vrålstad et al. 2018). Moreover eDNA metabarcoding is on the brink of successful implementation as standard biomonitoring tool for biodiversity research (Baird and Hajibabaei 2012).

eDNA technology can be used to implement the CBD Aichi Biodiversity Targets and the main aims of key biodiversity agreements. The main objective of the Birds Directive (2009/147/EC), the Habitats Directive (2006/105/EC) and CITES (or corresponding EU regulations) is to protect habitats and rare and endangered species. By assessing species/community attributes such as abundance, distribution or community composition and spatiotemporal changes, eDNA methodology can support the following management decisions:

1. Find, establish and monitor appropriate habitats for conservation measures, e.g. Natura2000 protection sites (e.g. Civade et al. 2016)
2. Evaluation of ecosystem and habitat condition (e.g. Bourlat et al. 2013)
3. Search for suitable reintroduction sites for native species
4. Monitoring of reintroduction attempts (e.g. Rojahn et al. 2018)
5. Investigation of the influence of natural or anthropogenic stress (e.g. deteriorated or polluted areas, climate change) on endangered species or communities
6. Restoration of terrestrial and aquatic habitats (e.g. Fernandes et al. 2018)
7. Evaluation of population parameters (e.g. recruitment rate) to control exploitation of wildlife populations

The Water Framework Directive (2000/60/EC) and the Marine Strategy Framework Directive (2008/56/EC) aim to reach a good ecological status in freshwater and marine systems. In assessing the biological status (e.g. species abundances, community structure), eDNA methodology can make valuable contributions to the conservation of aquatic environments (Hering et al. 2018). This could be achieved with a real-time eDNA biodiversity surveillance of endangered species and stressed communities. Many water courses in Germany are already equipped with river surveillance stations to monitor water levels, temperature and water quality. Thus temporary or permanent eDNA monitoring (e.g. measurements of abundance, biomass, community structure) could be easily implemented at these sites. Automated eDNA sampling stations with real-time data transmission (Bohmann et al. 2014) could give valuable information in changes of species abundances and composition. This approach could also serve as early warning system for the introduction and spread of alien invasive species.

In general, eDNA technology can be a practical tool to identify non-indigenious species at several steps of invasion (Comtet et al. 2015). The IAS regulation (EU) 1143/2014 aims to identify, control and eradicate invasive alien species, which pose a threat for EU

biodiversity. Conventional monitoring methods alone provide only scarce and incomplete inventories of IAS (Strayer et al. 2010). Moreover there is often a lag time between the arrival of IAS and visible ecological effects on native species and habitats (Crooks 2005). Thus invasive species are only detected at high population densities with conventional methods (Adrian-Kalchhauser and Burkhardt-Holm 2016). Species-specific eDNA marker systems enable to detect invasive species in early stages of invasion (e.g. Ardura et al. 2015, Goldberg et al. 2013, **Publication III**). eDNA methods can also be useful to assess the efficacy of protection measures such as barriers (e.g. Jerde et al. 2011) or eradication attempts (e.g. Davison et al. 2017, Miralles et al. 2016). Additionally, continual eDNA monitoring of previously invaded areas (e.g. intervals of month or years) could help to support restoration measures of natural habitats and communities.

In conclusion, eDNA methodology will make important contributions to biodiversity protection. Further advances in sampling, extraction, qPCR/sequencing technology and bioinformatic analysis will lead to an increasing application in ecology and wildlife monitoring. Combined approaches of eDNA and conventional monitoring procedures will lead to a more comprehensive understanding of biodiversity and ecosystem functioning.

This thesis, located at the interface between experimental and applied research, illustrates the suitability of the eDNA methodology in applied biomonitoring using the example of the water-borne pathogen *A. astaci*. The eDNA-based *A. astaci* detection was applied for the use in large-scale biomonitoring assessments and risk management. The application of eDNA methodology allowed to record spatially and temporally varying *A. astaci* sporulation dynamics. This technique also allowed to monitor the spread of *A. astaci*-positive NICS. There is no doubt that eDNA methodology will play an important role to safeguard native European crayfish species in the future.

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What has the PhD candidate contributed, and what have the coauthors contributed?

(1) Development and planning
Coauthors CN, SS and MT each 33%

(2) Implementation of the respective studies and experiments
PhD candidate: 100 % – field work (collecting water and tissue samples), molecular analysis

(3) Creation of the data collection and figures
PhD candidate: 100 % – created database, created figures

(4) Analysis and interpretation of the data
PhD candidate: 70% – analysis and interpretation of field and molecular data
Coauthors CN, SS and MT each 10% – contributed to data analysis and interpretation

(5) Writing the manuscript
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PRIMARY RESEARCH PAPER

eDNA-based crayfish plague monitoring is superior to conventional trap-based assessments in year-round detection probability

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Abstract The crayfish plague disease agent *Aphanomyces astaci* is a major threat to European crayfish populations, leading to mass extinctions when spores are transmitted into habitats of native species by infected invasive crayfish species. Current methods for detecting crayfish plague in carrier crayfish populations depend on time-consuming capture of individuals and screening via molecular methods. Highly sensitive environmental DNA (eDNA) methods are a promising tool for rapid and cost-efficient monitoring of pathogens in freshwater systems directly in water samples. For evaluating the usefulness of eDNA for *A. astaci* detection, the trap-based crayfish plague monitoring followed by qPCR screening of tissue samples was compared to an eDNA-based system to detect *A. astaci*- spores at a stream inhabited by an infected carrier crayfish population of *Pacifastacus leniusculus*. The presence of *A. astaci* was confirmed at all investigated sites with both sample types. Both methods showed comparable *A. astaci* prevalence, with the eDNA method being applicable across a longer annual time span, including winter, with greater reliability than the conventional method. Given the speed and reliability of the eDNA method for crayfish plague detection, this method might be the best choice for routine monitoring and evaluation of crayfish habitats to hinder the disease spread.

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Introduction

The oomycete *Aphanomyces astaci* (Verrucalvaceae, Beakes et al., 2014) causes the fatal crayfish plague among indigenous European crayfish species (Unestam, 1972). The crayfish plague disease agent poses a major threat to natural ecosystems, as it can drive entire native crayfish populations to extinction (Evans & Edgerton, 2002). Invasive North American crayfish species, e.g. the signal crayfish *Pacifastacus leniusculus*, were introduced to Europe in the late nineteenth century (Alderman & Polglase, 1988) and are known to be vectors of the pathogen (Unestam, 1972).

Aphanomyces astaci is transmitted by zoospores, which penetrate soft parts of the crayfish cuticle (Diéguez-Uribeondo et al., 2006). In invasive crayfish hosts, *A. astaci* hyphae are locally confined due to immune reactions leading to rapid melanization that is visualized as dark spots on the cuticle (Nyhlén & Unestam, 1980; Nyland & Westman, 1995). Native European crayfish species have developed similar immune response mechanisms, but suppression of the disease is not fast enough, thus hyphae spread throughout infected individuals leading to fatal mycosis (Unestam & Weiss, 1970). There is now accumulating evidence of a genotype-dependent virulence and thus partial resistance against some *A. astaci* strains found in native European crayfish species (Jussila et al., 2011; Viljamaa-Dirks et al., 2011; Makkonen et al., 2012; Kusar et al., 2013; Viljamaa-Dirks et al., 2013; Makkonen et al., 2014; Viljamaa-Dirks et al., 2016).

Crayfish plague ecology is studied intensively (e.g. Strand et al., 2011; Makkonen et al., 2013), but there are still many unresolved issues concerning *A. astaci* spread and sporulation dynamics in natural aquatic systems. Previous studies have shown that infected carrier crayfish pose a constant infection risk due to continuous release of *A. astaci* spores (Strand et al., 2012; Svoboda et al., 2013). The spread of the disease is facilitated by human-mediated actions, e.g. by accidental or intentional release of infected North American crayfish individuals into natural European crayfish habitats as a result of aquaculture trading or (often illegal) stocking actions, or by using contaminated fishing gear in different water courses in short succession (Alderman, 1996; Holdich et al., 2009).

If discovered, crayfish plague outbreaks are usually detected by observations of mortalities in native crayfish species with subsequent molecular diagnostics confirming the disease (OIE, 2012). For monitoring the distribution and infection levels of potential *A. astaci*- positive carrier populations, a commonly used procedure is a trap-based method, where crayfish are captured via trapping overnight to screen for *A. astaci* in tissue samples by molecular methods according to OIE standards (OIE, 2012). However, catch success with traps depends on various factors that are difficult to control—e.g. stream and river bed characteristics, size- and sex- selectivity and temperature-dependent activities of carrier crayfish (Abrahamsson, 1981; Reynolds & Matthews, 1993).

Strand et al. (2014) demonstrated that an environmental DNA (eDNA) approach is able to detect *A. astaci* spores directly from large freshwater systems. eDNA techniques are now a widely applicable tool to detect molecular evidence of invasive or endangered species in aquatic ecosystems directly from water samples (Thomsen & Willerslev, 2015). As eDNA detectability of aquatic species in freshwater systems has been found to surpass traditional monitoring methods (Jerde et al., 2011; Smart et al., 2015), this approach might be promising also for the routine detection of pathogens like *A. astaci*. Strand et al. (2014) found a positive correlation between *A. astaci* prevalence in signal crayfish populations, infection load in the crayfish tissue and the level of *A. astaci* spores in the lake water, suggesting a great potential for eDNA-based monitoring of crayfish plague. However, no direct comparison of methods detecting the crayfish plague via water or tissue samples has been done in terms of detection success and efficiency.

Thus, it was the aim of the current study to compare the performance of an eDNA-based detection protocol to the traditional trap-based method for *A. astaci* detection. In conjunction with this, the seasonal

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dynamics of spore release in natural water bodies throughout one whole year was of particular interest.

Materials and methods

Study site and field work procedures

Four sampling sites were chosen at the Ulmbach in the Westerwald region, a typical brook in the low mountain range in Hesse, Germany, with a total drainage area of 60.9 km^2 and a mean water flow of 741 l/s (Fig. 1). The stream is hosting a signal crayfish population with known *A. astaci* carrier status. We also tested two ponds (Mümling) with known *Astacus astacus* populations as negative control samples. Sampling took place once a month between April 2013 and March 2014 for a total of 12 sampling events per site over the course of a year. Permission for tissue and water sampling was obtained from local conservation authorities. All materials and equipment were thoroughly cleaned and disinfected with 0.25% peracetic acid and/or isopropanolic/ethanolic disinfectant after each sampling event.

Tissue sampling

Crayfish traps (type "Pirat", Engel-Netze GmbH & Co.KG, Germany) were used to catch signal crayfish and were deployed from April 2013 to March 2014 at each sampling site for one night using dog treat as a bait. Carapax size and sex of captured individuals were recorded. All captured signal crayfish (Table 1) were taken to the laboratory on ice and frozen at − 20 °C for further analysis.

eDNA sampling

Per sampling event in the stream Ulmbach, four independent eDNA samples were taken at each sampling site before the traps were placed in the water. Water samples were filtrated directly in the field through glass fibre filters (Ø 47 mm) with 2 µm pore size (Millipore, Merck KGaA, Germany) on in-line filter holders (Millipore, Merck KGaA, Germany) with a peristaltic pump system (Masterflex, Cole-Palmer Instrument Company, LLC, USA) according to Strand et al. (2014). Depending on sampling site, weather events, and seasonal variation of particle density, the sampled water volume varied from 1.6 to 10 l (Supplement Table 2).

Water samples from the negative control sites in the Mümling system were initially pumped from the

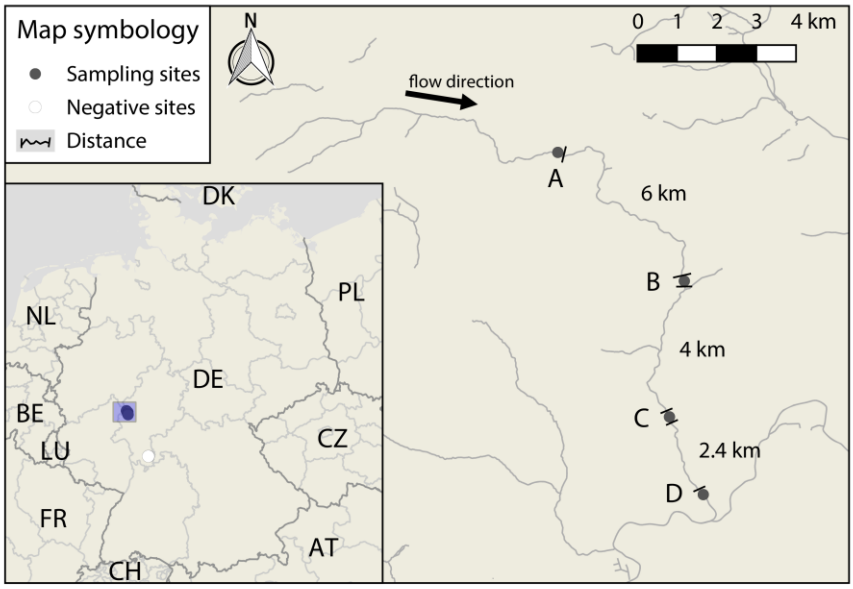

Fig. 1 Sampling locations targeted in this study. All samples were taken in Germany (DE) in the federal state Hesse in the stream Ulmbach (dark circles) with a known *A. astaci*- positive signal crayfish population and in two ponds of the Mümling water course (white circles) as negative control sites with *A. astaci*-negative noble crayfish populations. Exact positions of all six localities are given in Supplement Table 1

Table 1 Individuals trapped with number of traps in parentheses

Site	Sampling event											
	2013									2014		
	IV	V	VI	VII	VIII	IX	X	XI	XII	I	II	III
A	0 (2)	0 (2)	3 (2)	5 (2)	10 (3)	16 (5)	5 (4)	1 (4)	0 (4)	0 (4)	0 (4)	0 (4)
B	0 (3)	0 (1)	1 (3)	5 (3)	17 (3)	19 (5)	26 (4)	3 (4)	0 (4)	0 (4)	0 (4)	0 (4)
C	2 (3)	0 (2)	7 (3)	8 (3)	30 (3)	32 (5)	13 (6)	2 (6)	1 (6)	0 (6)	0 (6)	0 (6)
D	0 (2)	0 (2)	0 (2)	1 (2)	0 (3)	2 (5)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)	0 (4)

Trap numbers increased from September 2013 due to purchase of additional traps

bottom of the ponds with a bilge pump and prefiltered over a net cascade (between 20 and 100 µm, depending on turbidity) into 10 l- canisters prior to filtration over glass fibre filters. Due to high water turbidity at these sites, four filtration subsamples per site were produced to increase total water filtration volume, which were pooled after DNA extraction.

After filtration, glass fibre filters were transferred to 50 ml screw-cap tubes with sterile forceps, stored in a cool box ($\sim$ 4°C) to minimize microbial degradation, brought to the laboratory on the same day and stored at − 20°C until further processing. At each water sampling event, water temperature was recorded.

DNA extraction

Tissue extraction

Tissue samples of all captured crayfish were analysed. Parts of soft cuticle, tail fan and of a walking leg were used. DNA was extracted according to Vrålstad et al. (2009).

Glass fibre filter (GFF) extraction

Water samples from the year-round assessment were extracted according to Strand et al. (2014) with minor deviations. Addition of β-mercaptoethanol was omitted and no RNase treatment was conducted, as RNA did not interfere in qPCR analysis. Whereas Strand et al. (2014) used 2400 µl of the upper phase (2 × 1200 µl), we used just 1500 µl (2 × 750 µl) due to low phase formation. A second chloroform extraction step using 500 µl was carried out, with centrifugation at 12.000×*g* for 5 min. The upper phase, 600 µl, was transferred to a new tube and 900 µl isopropanol was used for DNA precipitation. All following steps followed the instructions of Strand et al. (2014). One DNA subsample was assayed in qPCR, since we assumed that spores on the filter and DNA in the aqueous phase are equally distributed and should lead to similar DNA concentrations in both subsamples.

During the analysis process we further optimized the extraction process for highly turbid waters like ponds and therefore a modified extraction protocol was used for the negative control sites, which allows the extraction of complete DNA from one glass fibre filter. For this purpose, the complete upper phase ($\sim$ 1500 µl) was transferred to a 5-ml reaction tube (Eppendorf AG, Hamburg, Germany). The subsequent extraction procedure followed the description above, except from using 1000 µl chloroform, 1 × 1200 µl supernatant, 1800 µl isopropanol, 600 µl EtOH (70%) and a speed of 3.005×*g* in all centrifugation steps. Since four filtration subsamples were produced at both negative control sites, DNA pellets were resuspended in 4 × 25 µl TE buffer (pH 8.0) with subsequent pooling of DNA extracts. In each extraction run, one extraction blank control (4 ml CTAB buffer) was included.

qPCR and data analysis

For the detection of *A. astaci* in tissue and water samples, the highly species-specific and -sensitive (Tuffs & Oidtmann, 2011) TaqMan® MGB qPCR Assay was used according to Vrålstad et al. (2009) with further adjustments in utilizing TaqMan® Environmental Master Mix 2.0 and slightly different temperature/time conditions with 62°C annealing for 30 s during qPCR procedure according to Strand et al. (2011, 2014). All samples were run for six times: 3× concentrated and 3× 10-fold diluted. All plates were run with four standard concentrations in duplicate serving as positive control and for calculating a

standard curve for all plates of the experiment. Also a NTC (PCR water) and a negative extraction blank control were included in each plate. *Ct* values were obtained with a TOptical Gradient 96 cycler (Biometra, Analytik Jena, Göttingen) and calculated with the software qPCRSoft 3.1 (Biometra, Analytik Jena, Göttingen). For relative quantification, Ct values for each sample were averaged, and PFU (PCR-forming unit) concentration per sample was calculated by using a master standard curve (formula: $y = (-1.633) \times \ln(x) + 41.232$; $r^2 = 0.99$; slope $= -3.7598$; efficiency 85%). eDNA samples showed negligible differences between diluted and undiluted replicates, therefore PFU values of the concentrated replicates were used for spore estimation. Tissue samples showed very high inhibition in concentrated replicates, requiring the 10-fold diluted replicates for estimating the PFU amounts. For water samples, the spore concentration (spores l^{-1}) was estimated according to Strand et al. (2014) and divided by the total amount of filtered water per sample. For tissue samples, PFU values were multiplied by 10 to account for the dilution factor. Only when a sample result (as mean value of all qPCR replicates) exceeded the assay-specific limit of detection (LOD) of 5 PFU, which corresponds to an infection level of $\geq A_2$ (Vrålstad et al., 2009), it was regarded as *A. astaci*-positive. Infection levels in this work refer to "agent levels" as used by Vrålstad et al. (2009). These are semi-quantitative categories based on the observed PFU values (PFU_{obs}), regarded as a measure of accessible target concentration.

Statistics

Statistical data analysis and graphical visualization was performed with the software GraphPad Prism, version 7.01 (GraphPad Software Inc., La Jolla California, USA, 2016). Catch Per Unit Effort (CPUE) was calculated with total number of captured signal crayfish divided by the amount of traps per night and per sampling site. For tissue samples, one-factor analysis of variance (ANOVA) and for water samples two-way ANOVA followed by Bonferroni's multiple comparison tests were applied to compare the mean PFU/spore amounts across sampling time and site.

Results

A. astaci prevalence and load in crayfish tissue

Crayfish CPUE showed distinct seasonal variation (Fig. 2). Trapping crayfish was most successful with rising water temperatures starting in June, reaching a maximum in August. With decreasing water temperatures catch success also decreased, and in the winter dormancy period between January and March 2014 no crayfish could be captured. The sex ratio of all captured crayfish over the year was 57.9% females to 42.1% males. Measured carapax length averaged 407 ± 0.71 mm for females and 416 ± 0.74 mm for males.

The observed prevalence of crayfish infected with *A. astaci* ranged from 68 to 100% in PCR assays, depending on the month (Table 2). Because of low catch success in April and December 2013, only one and two individuals, respectively, contributed to prevalence measurements. The PFU level median (with 25th and 75th percentile) in tissue samples (Fig. 3) increased from June steadily to August, reaching an annual maximum in September with 419 (219, 711) corresponding to an infection level of A_3. With decreasing water temperatures in autumn also PFU values decreased until November. Overall, of 209 crayfish individuals captured, 12% showed no or very low infection (A_0 and A_1) and 88% were *A. astaci*-positive with infection levels $\geq A_2$ (Table 2).

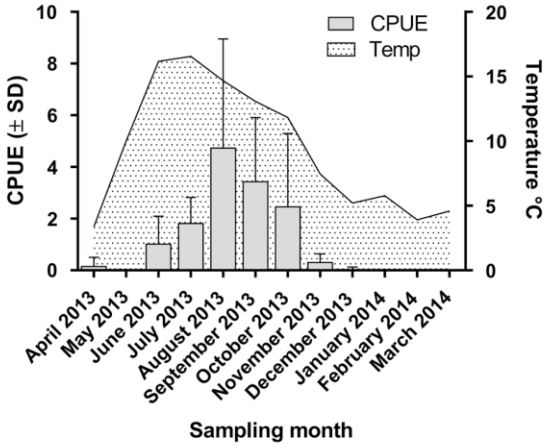

Fig. 2 Catch success of *Pacifastacus leniusculus* measured in CPUE (*catch per unit effort*) as mean of all sites with standard deviation with monthly water temperature (dotted background)

Table 2 Annual *A. astaci* prevalence in crayfish tissue samples (in % of positive samples) and infection levels based on *A. astaci* PFU (PCR-forming units) determined by qPCR analysis according to Vrålstad et al. (2009)

Sampling month	Individuals trapped	*A. astaci* prevalence	Infection levels					
			Negative		Positive			
			A_0	A_1	A_2	A_3	A_4	A_5
April 2013	2	100%	0	0	1	1	0	0
May 2013	–	–	0	0	0	0	0	0
June 2013	11	82%	2	0	8	1	0	0
July 2013	19	68%	6	0	9	4	0	0
August 2013	57	84%	7	2	16	32	0	0
September 2013	69	94%	4	0	3	59	2	1
October 2013	44	95%	2	0	5	35	2	0
November 2013	6	83%	1	0	1	4	0	0
December 2013	1	100%	0	0	0	1	0	0
January 2014	–	–	0	0	0	0	0	0
February 2014	–	–	0	0	0	0	0	0
March 2014	–	–	0	0	0	0	0	0
Total annual	209	88%	22	2	43	137	4	1

Values of tissue samples were combined for all sampling sites A–D

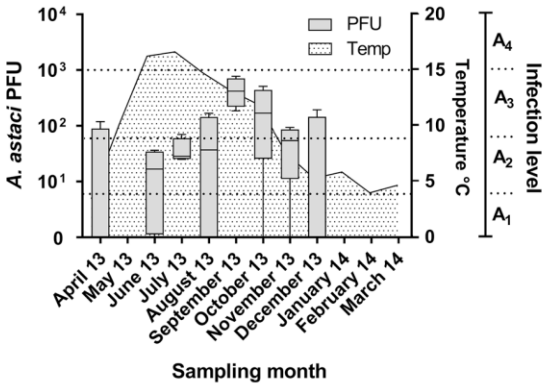

Fig. 3 Results of crayfish tissue samples, *A. astaci* PFU (PCR-forming units) and corresponding infection levels (according to Vrålstad et al., 2009) as mean of all sites with standard deviation with monthly water temperature (dotted background)

Aphanomyces astaci eDNA prevalence and load in water

Aphanomyces astaci DNA was detected throughout the year (Fig. 4) with eDNA samples at all sites, also during the cold season, with 80% of the 192 eDNA samples being *A. astaci*- positive (Table 3). The prevalence of positive *A. astaci* detections was correlated with the water temperature, as measured prevalence was less than 100% in April 2013 and November 2013 to March 2014, which also were the months with the lowest water temperatures. Spore amounts were significantly higher in June to October 2013 compared to all other months (April and May 2013: $P < 0.001$; November 2013 to March 2014: $P < 0.001$ to $P < 0.01$; two-way ANOVA). All sites exhibited a significant peak in October. In this month, eDNA samples of site C showed a significantly higher detection signal compared to all other sites (A and D: $P < 0.0001$, B: $P < 0.001$; two-way ANOVA). Additionally, eDNA samples taken from *A. astacus* habitats were tested consistently negative for *A. astaci* with infection levels of A_0 (data not shown).

Discussion

To our knowledge, this is the first study to directly compare the performance of an eDNA-based detection for *A. astaci* with a trap-based approach in a year-round assessment, thus allowing us to evaluate under which circumstances the one or the other method is superior.

Our results show a distinct seasonal variation in catch success with traps and *A. astaci* prevalence in eDNA samples. Crayfish trapping is generally conducted between May and October in Germany, because this fishing season is approved for a long time due to increased yield, and thus, this result is not surprising. The eDNA results are in line with observations made by Strand et al. (2014), who assumed

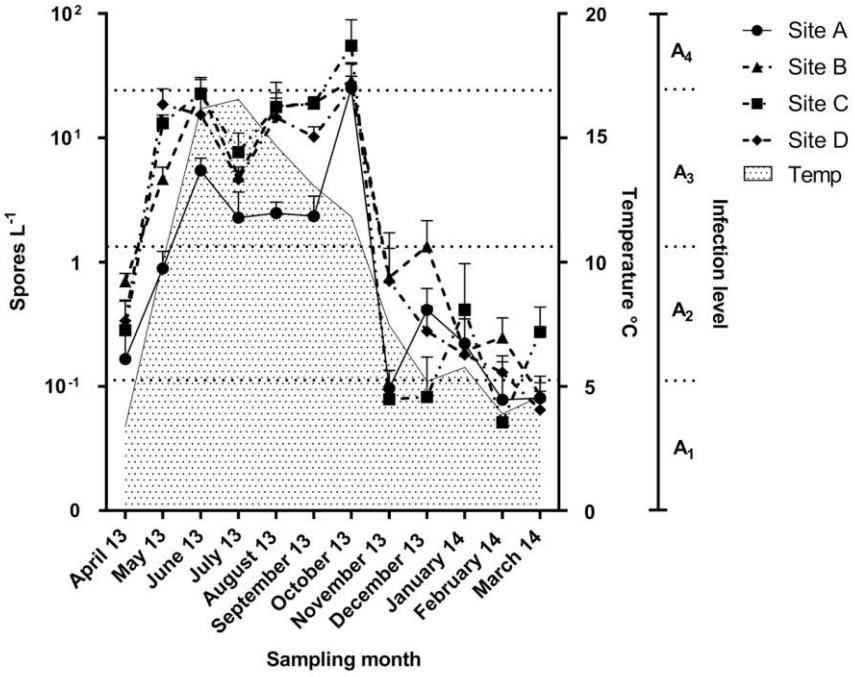

Fig. 4 Results of eDNA samples, with mean *A. astaci* spore estimate (± SD) per site and sampling month and corresponding infection levels, with monthly water temperature (dotted background)

Table 3 Annual *A. astaci* prevalence in eDNA samples (in % of positive samples) and infection levels based on *A. astaci* PFU (PCR forming units) determined by qPCR analysis according to Vrålstad et al. (2009)

Sampling month	# eDNA samples	*A. astaci* prevalence (%)	Infection levels					
			Negative	Positive				
			A_0	A_1	A_2	A_3	A_4	A_5
April 2013	16	88	0	2	12	2	0	0
May 2013	16	100	0	0	4	12	0	0
June 2013	16	100	0	0	0	10	6	0
July 2013	16	100	0	0	1	15	0	0
August 2013	16	100	0	0	1	7	8	0
September 2013	16	100	0	0	0	8	8	0
October 2013	16	100	0	0	0	9	7	0
November 2013	16	50	5	3	6	2	0	0
December 2013	16	81	0	3	10	3	0	0
January 2014	16	31	3	8	5	0	0	0
February 2014	16	50	2	6	8	0	0	0
March 2014	16	56	2	5	9	0	0	0
Total annual	192	80	12	27	56	68	29	0

Values of eDNA samples were combined for all sampling sites A–D

that spore concentrations could be subjected to seasonal variations due to their observations during a 25-week sampling period (between May and October). In this study, we were able to monitor the seasonally varying *A. astaci* spore amounts throughout the year and demonstrate that no time period can be proclaimed safe in terms of transmission risk because of a continuous release of *A. astaci* spores. Even in the winter season, the *A. astaci*- spore content is oscillating in low concentrations in water bodies.

Generally, the pathogen load in tissue samples and corresponding spore amounts in water samples are

dependent on the host species origin (invasive or native; e.g. Kozubíková et al., 2009; Pârvulescu et al., 2012), the disease process (acute or chronic; Alderman & Polglase, 1988) and life cycle stage (moult or intermoult; Strand et al., 2012) as well as the *A. astaci* genotype (Diéguez-Uribeondo et al., 1995), its virulence (Makkonen et al., 2014) and the overall spore challenge (Alderman & Polglase, 1988). The signal crayfish population under study was characterized by a high proportion of medium-level pathogen loads (A_3) in tissue samples throughout the year (Fig. 3), presumably due to an increased spore challenge in the ambient water as a result of a generally high *A. astaci* prevalence of 88% in the population.

The life-cycle stage of carrier crayfish, especially the moulting period, could have an effect on the measured *A. astaci* prevalence in water samples. Adult signal crayfish only moult once or twice a year in natural water bodies (Westman & Savolainen, 2002), solely in seasons of suitable water temperature, generally between April and October (Aiken & Waddy, 1992). The moulting frequency is mainly dependent on water temperature, food availability and population density (Aiken & Waddy, 1992; Guan & Wiles, 1999; Kozák et al., 2009; Lowery & Holdich, 1988). We assume synchronized moulting in favourable seasons (Aiken & Waddy, 1992) leading to an increased detection probability of *A. astaci* via eDNA method in the warmer season.

It was previously known that the peak activity of *P. leniusculus* was during summer due to elevated water temperatures, and very low activity was observed during winter months (Flint, 1977). In concordance, our results indicate a temperature-dependent activity of signal crayfish, the trap-based approach being the most successful in the period of the highest activity of crayfish, i.e. in the summer months. In the cold period (winter from January to March 2014) and in an uncommonly cold May 2013 ($\sim$ 10°C), the trap-based assessment likely failed due to low activity leading to no-capture result. This is also reflected by the need of applying many traps per site in months with low activity and population density in order to reach statistically sound numbers of trapped individuals. This increases material costs and time for placement of traps and decontamination of equipment. In the current study, some sites proved unfavourable for placing traps accurately, when the river bed was very shallow or deep, or when a high fluctuation of water flow was observed, which is typical for small mountainous catchments. These difficulties and uncertainties of the conventional trap-based monitoring obstruct reliable detection of *A. astaci*. In contrast, the eDNA method provides greater flexibility in the choice of sampling sites, because water samples can be taken from almost everywhere at a water course. The time saving and thus cost-efficient sample processing in the field and in the laboratory makes the approach highly recommendable for standard application in crayfish plague-monitoring programmes. The performance of the eDNA method in certain real-life situations—e.g. low infection prevalence or population density—when the spore amount in the ambient water body is reduced, should be further evaluated.

Water temperature seemed to influence the *A. astaci* prevalence in eDNA samples (Fig. 4), since it changed considerably during the year and is thereby influencing life history traits of the pathogen. Previous studies performed under laboratory conditions showed that spore release is modulated by water temperature, when significantly more spores are released from latent carrier crayfish at higher water temperatures of 18°C (Strand et al., 2012; Svoboda et al., 2013). Optimal growth and sporulation of *A. astaci* occur between 16 and 20°C (Alderman & Polglase, 1986; Diéguez-Uribeondo et al., 1995). In line with these findings, our results indicate a period of 7 months (April–October) with water temperatures between 12 and 16°C, when *A. astaci* DNA amounts in eDNA samples (Fig. 4) are high due to elevated spore production in natural water systems. Spore concentrations in water samples in October 2013 appear extraordinary high compared to the other months, because conditions in autumn are becoming increasingly less beneficial, through a combination of lower water temperatures and less light, thus the decreasing activity of crayfish gives reason to expect decreased *A. astaci* spore amounts in the water column. This observation can probably be explained by a synchronized moulting event of infected carrier crayfish just before the unfavourable and growth-inhibiting winter period starts (Aiken & Waddy, 1992; Lowery, 1988), which leads to higher infection rates due to the softer cuticle that favours infections. Additionally or alternatively, this rise of spore concentration might coincide with the mating period of crayfish, which is commonly restricted to less than 1 month between

October and November (Abrahamsson, 1971; Gherardi, 2002), when water temperature drops to 10–15°C. Mating is often accompanied by heavy injuries and also increased female mortalities due to aggressive mating behaviour (Mason, 1970; Woodlock & Reynolds, 1988; Gherardi, 2002). Lost limbs and dead crayfish are very likely to increase the release of *A. astaci* spores from the decaying crayfish materials, thereby increasing the detection probability of spores in the ambient water.

In many cases, eDNA approaches led to high detection probabilities of a variety of aquatic species in freshwater systems, implying to be more sensitive than traditional methods (examples Jerde et al., 2011; Smart et al., 2015; references in Thomsen & Willerslev, 2015). While the overall detection rate in tissue samples was slightly higher with 88% compared to eDNA samples achieving 80%, it should be emphasized that the period for reliable *A. astaci* detection with tissue samples was limited to a period of 6 months (June–November 2013), whereas detection with eDNA samples was possible throughout the complete year and with less effort in terms of time and costs. Even though our method yielded good results, other possibilities are reported, which could lead to further improvements of the eDNA method in using different filter types and pore sizes, filtered water volumes or DNA extraction methods (e.g. Goldberg et al., 2011; Jerde et al., 2011; Takahara et al., 2012; Thomsen et al., 2012). Although density dependence and differing environmental conditions influencing the detectability of target DNA are still a concern in eDNA studies (Barnes & Turner, 2016; Goldberg et al., 2016), we are confident that the eDNA method for crayfish plague detection is a useful substitution method for tissue-based assessments in the future.

Conclusion

Our results suggest that the eDNA method has several advantages over the traditional trap-based assessments. The eDNA method used in this study showed similar detection rates, a wider time period for detection throughout a year, greater flexibility in choice of sampling sites and a decreased time/cost effort in field and lab work. This renders this approach recommendable for use in large-scale crayfish plague-monitoring programmes. Nevertheless, more research is needed especially focusing on the distribution and population density of infected carrier species, before solely the eDNA approach could be used to test natural water courses for the presence or the absence of *A. astaci*. Furthermore, future research could focus on alternative eDNA protocols that can increase the detection rates of *A. astaci* spores in natural water courses.

Our findings also suggest that no time period can be proclaimed safe of *A. astaci* transmission risk because of a continuous release of zoospores.

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Compliance with ethical standards

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

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

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Publication I – Supplementary Material

Supplement Table 1: Sample site coordinates

Site		Coordinates
positive	A	N50.60990 E8.25585
	B	N50.58123 E8.30114
	C	N50.55025 E8.29632
	D	N50.53248 E8.30900
negative	Neg1	N49.705778 E8.952222
	Neg2	N49.703111 E8.943694

Supplement Table 2: Filtered water volumes (in L) per individual eDNA sample. Volumes marked with * were achieved with four pooled subsamples.

Site	Sampling event											
	2013									2014		
	IV	V	VI	VII	VIII	IX	X	XI	XII	I	II	III
A	5	4	7	5	4	5	5	10	8.5	5	8.5	10
B	10	4	10	10	10	10	5	7.5	10	5	8.5	10
C	7	1.6	7	7	10	10	5	7.5	10	4	7	10
D	7	1.6	3.5	7	7.5	10	5	7	8.5	4	7	10
Neg1										1.6*		
Neg1										2.5*		

Publication II

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Declaration on the contributions of authors

What has the PhD candidate contributed, and what have the coauthors contributed?

(1) Development and planning
Coauthors CN, SS and MT each 33%

(2) Implementation of the respective studies and experiments
PhD candidate: 100% – field work (collecting water and tissue samples), molecular analysis

(3) Creation of the data collection and figures
PhD candidate: 100% – created database, created figures

(4) Analysis and interpretation of the data
PhD candidate: 80% – analysis and interpretation of field and molecular data
Coauthor SS 10% – contributed to data analysis and interpretation
Coauthors CN and MT each 5% – contributed to data analysis and interpretation

(5) Writing the manuscript
PhD candidate: 80%
Coauthor SS: 10%
Coauthors CN and MT each 5%

Date/place: _____ / Frankfurt am Main, Germany

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Affirmative confirmation of the above information:

Date/place: _____ / Frankfurt am Main, Germany

Signature PhD advisor: _____

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Comparison of two water sampling approaches for eDNA-based crayfish plague detection

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ABSTRACT

The crayfish plague agent *Aphanomyces astaci* causes high fatality rates among European crayfish species and is transmitted by semi-immune North American crayfish species via zoospores. Recently environmental DNA (eDNA) techniques have been developed to detect the pathogen directly in water samples.

To identify the optimal technique for concentrating spores out of water samples we tested two water filtration methods, namely depth filtration (DF) and dead-end ultrafiltration (DEUF), with subsequent qPCR-based detection of *A. astaci* spores from the water column in three river systems in Germany.

Both eDNA methods were successful in recovering and detecting *A. astaci* spores from all three lotic water systems and the detection patterns were generally consistent across watercourse and season. Water turbidity negatively affected the *A. astaci* spore detection with both eDNA methods, with increasing pellet weights for the DEUF method and decreasing water volumes for the DF samples. Although filtering high-volume water samples with the DEUF method led to slightly higher detection rates of *A. astaci* and seemed to be more sensitive in *A. astaci* detection, its application is highly laborious and more costly. We therefore propose to use the DF method for large-scale screenings of *A. astaci* in running waters due to its fast, cost-effective and easy-to-apply sample processing and the very robust quantification results. We are confident that this method might be favored as well for eDNA studies of other organisms.

1. Introduction

In recent years the increasing use of eDNA (environmental DNA) methods in conservation biology and environmental biomonitoring led to the detection of genetic traces of divers organisms in various environments (Taberlet et al., 2012a; Ficetola et al., 2008; Thomsen et al., 2012a; Thomsen and Willerslev, 2015) using different sampling and DNA extraction methods (Rees et al., 2014). These methods proved highly suitable for the detection and monitoring of invasive species (e.g. Dougherty et al., 2016) and associated parasites (Bass et al., 2015).

eDNA-based detection methods focussing on particular species require the development of specific field and laboratory protocols which consider a species' preferred habitat conditions – e.g. hidden in sediments/shelters, preference of slow of fast flowing stream sections – to increase the detection probability (Barnes and Turner, 2016; Deiner et al., 2015; Goldberg et al., 2016). The considerable diversity of these protocols makes it hard to compare the detection rates of different eDNA methods in aquatic systems (overview over taxa and habitats in Thomsen and Willerslev, 2015). Adequate water sampling procedures in the field are of prime importance for eDNA studies, since different methods can affect sensitivity and robustness of single species detection and eDNA metabarcoding approaches based on next generation sequencing (NGS, Lodge et al., 2012; Taberlet et al., 2012b; Thomsen et al., 2012b; Valentini et al., 2016). The lack of comparability studies (Barnes and Turner, 2016) emphasizes the need to test different field procedures in terms of detection probabilities, application in

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conservation issues, handling and method-specific drawbacks to enable to select the most promising water filtering procedure for the specific scientific purpose.

Diverse water sampling techniques are currently available, which are modified for use in standing and running waters. eDNA sample processing can be based on two different strategies, namely precipitation and filtration (Goldberg et al., 2016). For precipitation, the volume of water samples is restricted to very small amounts of usually up to 15 ml (e.g. Dejean et al., 2012; Ficetola et al., 2008). In small and/or lentic water bodies like ponds with high population densities of the target organism, this method can already lead to high detection success. Filtration procedures are mostly applied in large and/or lotic water bodies (e.g. Goldberg et al., 2011; Jerde et al., 2011), which allows larger water volumes to be processed. Species detection via eDNA in these water bodies can be a difficult task due to heterogeneously distributed eDNA particles by non-homogenous water mixing (Mahon et al., 2013, Turner et al., 2014), fast velocities and therefore short water retention time near the target organism as well as environmental factors determining eDNA persistence in aquatic systems (Barnes et al., 2014). Within the field of eDNA research, a vast variety of different filter types are used for the detection of target species in water samples via filtration. These include glass fiber filters (Agersnap et al., 2017; Jerde et al., 2011; Strand et al., 2014), nylon filters (Thomsen et al., 2012b), cellulose nitrate filters (Goldberg et al., 2011), polycarbonate filters (Takahara et al., 2012) or Sterivex™- GP filters (Agersnap et al., 2017; Spens et al., 2017). The choice of filter type is mainly dependent on the eDNA particle size of interest (intracellular/sediment-bound or extracellular/free-floating) and filtration capacities usually range from 15 ml to 10 l, mostly depending on filter media properties such as pore size or hydrophilicity. Each filter type is treated differently during sample processing, because filter media characteristics are considered in DNA extraction protocols. Furthermore, water turbidity is a major issue, leading to fast clogging of filter media, and this issue becomes more problematic with decreasing pore sizes.

Processing large-volume water samples is beneficial for detecting rare as well as recently invaded species, which occur in low densities in natural water systems (Wilcox et al., 2013). For concentration of microbes in large-volume water samples and subsequent detection of target species, different filtration methods are available, including depth filtration (DF) and dead-end ultrafiltration (DEUF; e.g. Strand et al., 2014). The DF approach is based on filtration of water over porous and high-pressure compatible multilayer depth filters, which allows for retaining many particles of various sizes on the surface and inside the filter media before becoming clogged (Sutherland and Chase, 2008). For eDNA studies, the use of glass fibre filters meets optimal conditions for recovery of divers particle sizes from water samples and obtainable from different brands with pore sizes between 0.7–2.7 µm.

The DEUF method uses ultrafilters, where the water flow runs vertically through a hollow fibre matrix inside of an ultrafilter cartridge, and is nowadays often used for processing large water volumes of up to 100 l for concentration and recovery of bacteria and other microbes (Kearns et al., 2008; Leskinen and Lim, 2008; Leskinen et al., 2009; Mull and Hill, 2012; Smith and Hill, 2009).

Since DF and DEUF sampling procedures are both applicable in eDNA studies in aquatic systems, we aimed to test which method is more sensitive, straight-forward and feasible, in particular in respect to the detection of invasive species or pathogens. For this we chose the crayfish plague agent *Aphanomyces astaci* (Schikora, 1906), which elicits the fatal disease among susceptible crayfish species (Unestam, 1972) and now occurs throughout the whole European continent due to the invasion of semi-immune carrier crayfish species like the signal crayfish *Pacifastacus leniusculus* (Dana, 1852; Crandall, 2017; Souty-Grosset et al., 2006). Most indigenous crayfish species are now rare and endangered due to their vulnerability to *A. astaci* infection (Alderman and Polglase, 1988; Holdich et al., 2009). *A. astaci* is a water borne oomycete pathogen and is transmitted via motile zoospores (Alderman and Polglase, 1986) from infected hosts, which are continually released into the ambient water (Strand et al., 2012; Svoboda et al., 2013).

Zoospores were successfully detected with eDNA techniques based on DF and DEUF directly from water samples (Strand et al., 2011, 2012, 2014). Both techniques were equally sensitive to detect *A. astaci* spores despite the much larger water volume sampled with the DEUF method (Strand et al., 2014).

Here we aimed to evaluate if sampling of large water volumes increases the detectability of *A. astaci* in three river systems with *A. astaci*-positive crayfish populations. Our focus was to thoroughly examine the method-specific handling, application and difficulties in the field and in the laboratory as well as the dependence on season. Our overall aim was to identify the best procedure for the monitoring of *A. astaci* in natural water bodies based on eDNA.

2. Materials and methods

2.1. Study sites

Three test streams with known *A. astaci*-positive populations of the signal crayfish were selected: The Ulmbach (total length: 23 km, drainage area: 61 km^2, mean discharge; 741 Ls-1), the Lütter (total length: 18 km, drainage area: 51 km^2, mean discharge: 672 Ls-1) and the Weschnitz (total length: 58 km, drainage area: 148 km^2, mean discharge: 2116 Ls-1). Prior to the eDNA application the distribution range of *A. astaci*-positive signal crayfish was surveyed and eight sampling sites (A–H) were chosen at each test stream (Fig. 1, Supplement Table 1). All streams show typical features for the Central European low mountain region, with siliceous, brash and stone-rich areas as well as alternating velocity. Sampling events took place in May ('spring') and August ('summer') 2013 and permission was granted by local conservation authorities. All materials, surfaces and equipment were thoroughly cleaned and disinfected with 0.25% peracetic acid and/or isopropanolic/ethanolic disinfectant after each sampling event and between sampling areas. At each water sampling event the local water temperature was recorded.

2.2. Sampling procedure

Water samples for the DF and DEUF method were processed in parallel directly in the field (Fig. 2). For the DF setup, four water samples were collected per sampling site according to the water sampling procedure of Wittwer et al. (2017). Filtered water volume ranged between 1.6 to 10 l, depending on the water turbidity (Supplement Table 2). For the DEUF setup, Asahi Kasei Rexeed™ 18UX dialyse filters (Diamed Medizintechnik, Germany) with 1.8 m^2 surface area, an inner fibre diameter of 185 µm and a molecular cutoff of 30 kDa pore size were used with one filter cartridge per site. Prior to filtration, the ultrafilter matrix was prepared using 1 l of blocking buffer consisting of 0.01% sodium polyphosphate according to Hill et al. (2005). The DEUF setup of Smith and Hill (2009), as modified by Strand et al. (2014), was used to filter 100 l of water over one Rexeed™ ultrafilter per site in about 60 min with a total pumping flow rate of 1.6 l/min. Processed glass-fibre filters were transferred to 50 ml screw-cap tubes and the inlets/outlets of the processed ultrafilters were properly sealed. All samples were kept on ice until transfer to the laboratory, where DF samples were stored at −20 °C until further processing. Ultrafilter samples were eluted from the ultra-filter matrix using 400 ml of an elution buffer consisting of 0.5% TWEEN® 80 solution, 0.01% sodium polyphosphate and 0.001% Dow Corning® antifoam RD emulsion according to Hill et al. (2005). With backward flushing the ultrafilter content was pressed into sterile vessels using a peristaltic pump, resulting in approx. 600 ml eluate. The eluate from ultra-filter samples were collected in 50 ml screw-cap tubes and stored at −20 °C until processing. For extraction, 4 × 50 ml subsamples of the eluate were used. Assuming a 100% recovery rate of *A. astaci* spores from the

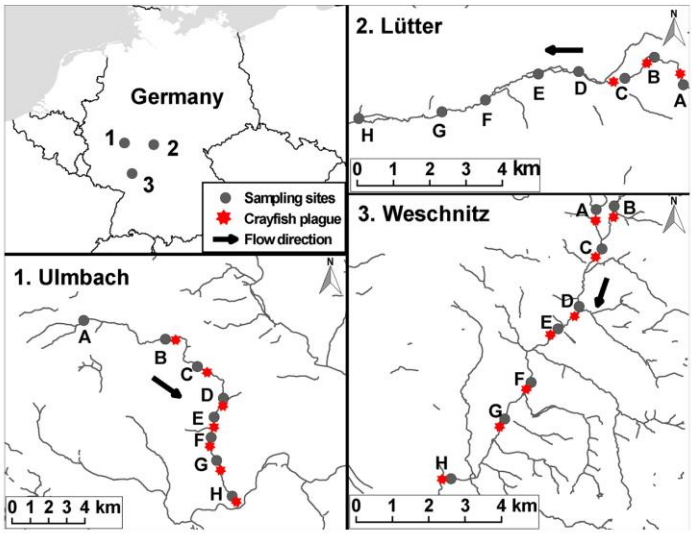

Fig. 1. Sampling areas. eDNA samples were taken in three regions of Hesse/Germany in the streams Ulmbach (1), Lütter (2) and Weschnitz (3) with known *A. astaci*- positive signal crayfish populations. Coordinates of all sampling sites are given in Supplement Table 1.

ultrafilter matrix, these subsamples represent the spore content in approximately ~8.333 l of filtered water. Water filtration capacity of the DF samples and the pellet weight of the DEUF samples were used as proxies for water turbidity.

2.3. DNA extraction

eDNA samples were extracted in a clean lab under rigorous standards to avoid cross-contamination.

DNA from glass fibre filters derived from DF sampling was extracted using a CTAB protocol according to Strand et al. (2014) with further

Fig. 2. Water sampling procedure. For the DEUF method (blue) the preparation of the ultrafilter matrix with blocking buffer was carried out prior to filtration. After filtration ultrafilter cartridges were eluted, the eluate recaptured in a vessel, subdivided in 12 × 50 ml screw-cap tubes and stored at −20 °C until further processing. For the DF method (orange) no pre- and postprocessing steps are necessary. Water filtration in the field with parallel DF and DEUF setup (middle). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

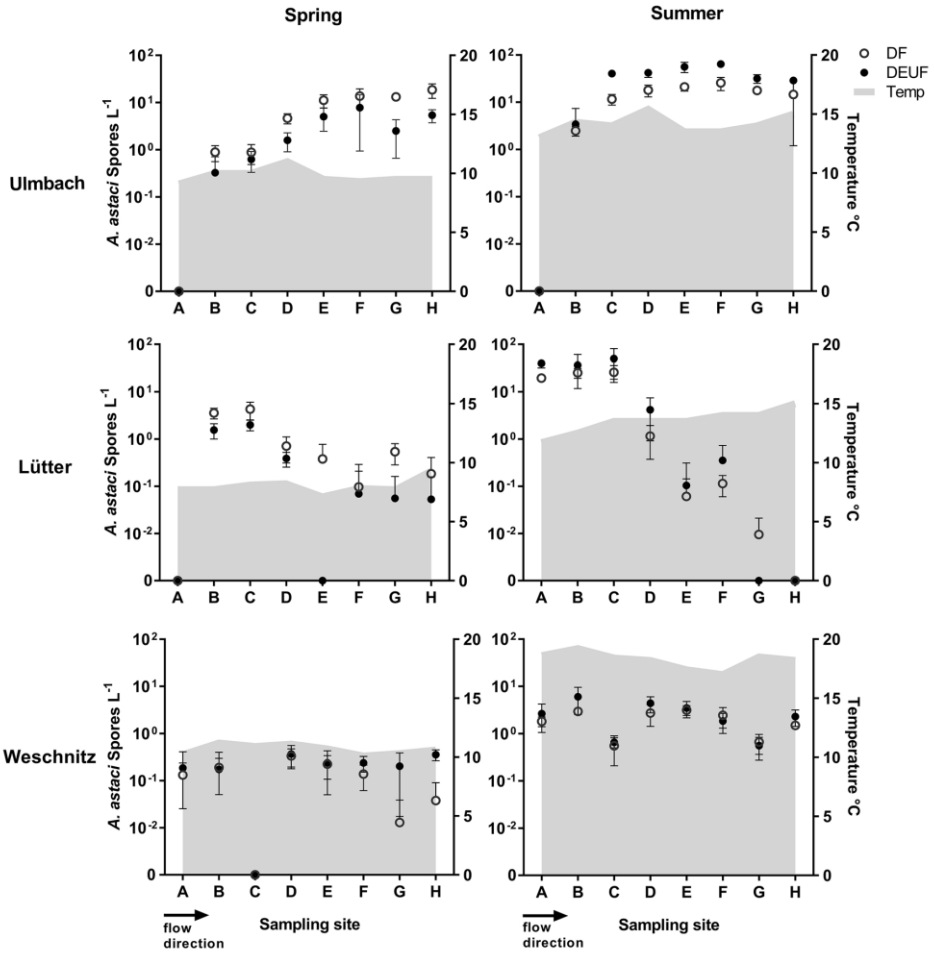

Fig. 3. Results of water sampling methods (DF = depth filtration; DEUF = dead-end ultrafiltration), with mean *A. astaci* spore estimate (± SD) per site, sampling month and water temperature (grey background).

modifications as described by Wittwer et al. (2017). One extraction blank control consisting of 4 ml CTAB buffer was included in each DF extraction run.

Frozen DEUF samples were allowed to thaw at room temperature and then centrifuged for 10 min at 3005*g* at room temperature. The supernatant was discarded and the pellet was transferred to a new tube. Afterwards, an additional centrifugation step for 5 min at 12.000*g* was carried out and the supernatant was discarded again. The complete resulting pellet was used for DNA extraction. Pellets weighed between 46–420 mg, depending on turbidity of the water at the respective sampling site (Supplement Table 3). The DEUF extraction protocol followed the description of Wittwer et al. (2017), except from using 1200 µl CTAB buffer, 20 µl proteinase K (20 mg/ml), 1000 µl chloroform, 1 × 800 ml supernatant and 1200 µl isopropanol. In each DEUF extraction run one extraction blank control consisting of 1200 µl CTAB buffer was included.

2.4. Quantification

The species-specific and sensitive qPCR assay of Vrålstad et al. (2009) was used to detect *A. astaci* in both sample types. Further improvements in sensitivity were achieved by using TaqMan® Environmental Master Mix 2.0 and a modified cycling protocol with annealing at 62 °C for 30 s (Strand et al., 2014). All samples were run with six replicates (3 x concentrated, 3 × 10-fold diluted). All 96-well plates were run with four standard concentrations obtained from *A. astaci* tissue samples in duplicate and served as positive control. Standards were used to calculate a master standard curve (formula: $y = (-1.633)*\ln(x) + 41.232$; $r^2 = 0.99$; slope = −3.7598; efficiency 85%) as inherent part of a complete dataset obtained during a large-scale project (grant F7/2012; Wittwer et al., 2017; C. Wittwer, unpublished data). Extraction blanks and NTC (water) controls as negative PCR controls were run on each plate to control for potential contamination. *A. astaci* quantification was performed on a TOptical Gradient 96 system (Biometra, Analytik Jena, Göttingen) and Ct values were calculated with the software qPCRSoft 3.1 (Biometra, Analytik Jena, Göttingen). PFU (PCR forming units) concentration was calculated by using the master standard curve mentioned above. Obtained PFU values were converted to spore amount estimates according to Strand et al. (2014) and divided by the total amount of filtered water per sample. Since DF samples showed negligible differences between diluted and undiluted replicates, PFU values of the concentrated

Table 1
A astaci detection in DF and DEUF samples (in% of positive samples) and spore concentrations ranked into pathogen levels based on *A. astaci* PFU (PCR forming units) determined by qPCR analysis according to Vrålstad et al. (2009).

Location	Season	# Water samples	DF					Detection	DEUF					Detection
			Pathogen level						Pathogen level					
		(DF + DEUF)	Negative		positive				negative		positive			
			A0	A1	A2	A3	A4		A0	A1	A2	A3	A4	
Ulmbach	spring	2 × 32	4	0	7	21	0	88%	6	0	5	20	1	81%
	summer	2 × 32	4	0	1	12	15	88%	6	0	0	2	24	81%
Lütter	spring	2 × 32	9	8	12	3	0	47%	17	0	8	7	0	47%
	summer	2 × 32	8	4	5	3	12	63%	12	0	4	4	12	63%
Weschnitz	spring	2 × 32	11	11	10	0	0	31%	9	0	23	0	0	72%
	summer	2 × 32	0	0	15	17	0	100%	0	0	7	25	0	100%
Total		**2 × 192**	**36**	**23**	**50**	**56**	**27**	**69%**	**50**	**0**	**47**	**58**	**37**	**74%**

replicates were used for spore estimation. DEUF samples showed very high inhibition in concentrated replicates, requiring the 10-fold diluted replicates for estimating the spore concentration, with the dilution factor subtracted out in the following analysis. The assay-specific limit of detection (LOD) was previously determined by Vrålstad et al. (2009) as 5 PFU and the limit of quantification (LOQ) as 50 PFU per qPCR reaction. All averaged quantification results exceeding the PFU value/spore estimate equal to the pathogen levels of $\geq A_2$ (as described in Vrålstad et al., 2009) were regarded *A. astaci*-positive (Supplement Fig. 1).

2.5. Statistics

Statistical data analysis and graphical visualization was conducted with the software GraphPad Prism version 7.01 (GraphPad Software Inc., La Jolla California, USA, 2016). Two factor analysis of variance (ANOVA) with Bonferroni's multiple comparison tests was performed on the dataset of each individual water course to compare mean spore amounts across season and method. Unpaired, two-tailed *t*-tests were performed on the measured *A. astaci* spore amounts of all sampling sites to evaluate the method-specific performance. For comparing the detection success of each eDNA method as a function of water turbidity, a linear regression was performed on measured spore amounts. For sensitivity testing, a nonlinear regression was performed on the obtained pathogen levels of both eDNA methods. The statistical significance was set at $\alpha = 0.05$.

3. Results

Both water sampling methods were able to detect *A. astaci* spores in all tested streams and both seasons (Fig. 3, Table 1). The 2-way ANOVA analysis revealed that the independent factors 'Season' and 'Method' had a significant effect on the detectability of *A. astaci*, with the factor 'Season' generally contributing more to the total variance and interactions between both factors were observed (Table 2). Both eDNA methods were able to detect *A. astaci* spores within the initially surveyed *A. astaci*- positive signal crayfish distribution range of the individual stream. The observed *A. astaci* detection patterns of both water sampling methods were generally consistent across water course and season (Fig. 3). In all water courses, a seasonal temperature increase resulted in increased *A. astaci* detection rates with both eDNA methods. Overall, of 192 samples taken for each water sampling strategy, 69% of all DF samples and 74% of all DEUF samples were *A. astaci*- positive (Table 1).

The observed detection rates substantially varied between seasons for both eDNA methods when comparing the spore amounts of all sampling sites (Fig. 4). The *A. astaci* spores L^{-1} median (with 25th and 75th percentile) in DF samples significantly increased from spring with

Table 2
Results of two- way- ANOVA for *A. astaci* spores L^{-1} of the two water sampling methods in sampling months May and August at the streams Ulmbach, Lütter and Weschnitz with DF = Degrees of Freedom, SS = Sum of Squares, MS = Mean Squares, F- values, variance levels and significance levels * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$.

Stream	Source of variation	DF	SS	MS	F- value	Variance [%]	p- value	Significance
Ulmbach	Season	1	17209	17209	65.76	26.13	< 0.0001	****
	Method	1	6090	6090	23.27	9.246	< 0.0001	****
	Season * Method	1	10121	10121	38.68	15.37	< 0.0001	****
	Residual	124	32449	261.7				
	Total	127	65870					
Lütter	Season	1	6663	6663	22.31	14.38	< 0.0001	****
	Method	1	1203	1203	4.028	2.597	0.0469	*
	Season * Method	1	1423	1423	4.763	3.071	0.0310	*
	Residual	124	37035	298.7				
	Total	127	46324					
Weschnitz	Season	1	216.2	216.2	82.02	36.74	< 0.0001	****
	Method	1	26.98	26.98	10.24	4.586	0.0017	**
	Season * Method	1	18.38	18.38	6.972	3.123	0.0093	**
	Residual	124	326.9	2.636				
	Total	127	588.5					

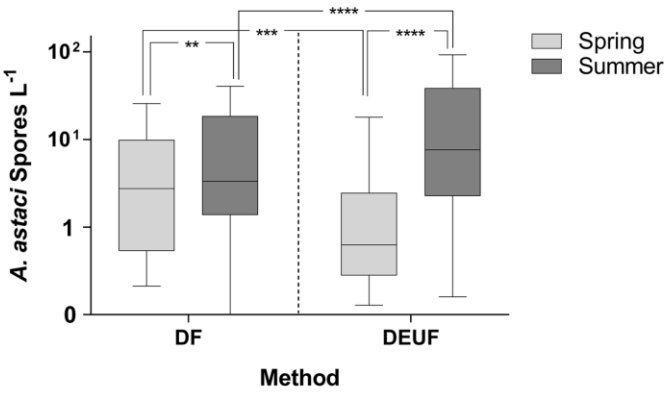

Fig. 4. Overall comparison of detectable *A. astaci* spores L^{-1} with the DF and DEUF method.

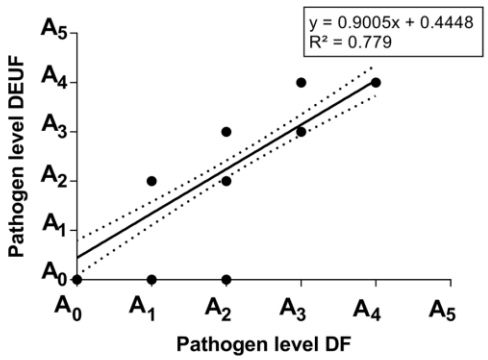

Fig. 5. Comparison of achieved pathogen levels of both water sampling methods over all sampling sites.

Table 3
Significant results of two- way- ANOVA post hoc tests (Bonferroni) for *A. astaci* spores L^{-1} of the two water sampling methods in spring and summer season at the streams Ulmbach, Lütter and Weschnitz with significance levels * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$.

Stream	Season	Method	t- value	p- value	Significance
Ulmbach	spring vs. summer	DEUF	10.13	< 0.0001	****
	summer	DF vs. DEUF	7.809	< 0.0001	****
Lütter	spring vs. summer	DEUF	4.883	< 0.0001	****
	summer	DF vs. DEUF	2.962	< 0.05	*
Weschnitz	spring vs. summer	DF	4.537	< 0.0001	****
	summer	DEUF	8.271	< 0.0001	****
	summer	DF vs. DEUF	4.129	< 0.001	***

~2.7 (0.5, 9.9) spores L^{-1} to summer with ~3.3 (1.4, 18.5) spores L^{-1} ($p < 0.01$; unpaired, two-tailed *t*-test). For DEUF samples a spore content of ~0.6 (0.3, 2.7) spores L^{-1} was measured in spring and detection rates increased significantly in summer with ~7.5 (2.3, 39.7) spores L^{-1} ($p < 0.0001$; unpaired, two-tailed *t*-test). Comparing the detection success of both eDNA methods, the DF method performed better in spring ($p < 0.0001$; unpaired, two-tailed *t*-test), whereas the DEUF method detected significantly more spores in summer ($p < 0.0001$; unpaired, two-tailed *t*-test). In terms of achieved pathogen levels our results indicate that the DEUF method was slightly more sensitive in *A. astaci* spore detection (Fig. 5), with observed pathogen levels being one magnitude higher than pathogen levels obtained with the DF method (Table 3).

Seasonal changes in water turbidity or suspended matter concentration in the tested streams had a major impact on filtration capacity of both water sampling methods. Increased amounts of suspended particles were observed in spring compared to summer (Fig. 6), reflected by the amount of filtered water through glass fibre filters (sample mean spring: filtration volume ~2.8 l; summer: filtration volume ~7.7 l) and also by the weight of pellets from DEUF extraction (spring: 245 ± 118 mg, summer: 85 ± 37 mg). Water turbidity was found to affect the *A. astaci* spore detection, with significantly higher detection probabilities reached with increasing water volume for DF samples and decreasing pellet weights for DEUF samples (Fig. 7).

The handling of the DF method was generally more efficient and straightforward compared to the DEUF method (Table 4). The DF method facilitates a straightforward water filtration and laboratory procedure without pre- and post-processing steps (blocking, elution, dilution), which considerably accelerates the speed of eDNA sample processing. The DEUF method requires more time for additional pre- and post-processing steps in addition to at least 60 min per sampling event. At the same time more consumables (reaction tubes, chemicals) and expensive ultrafilter cartridges (~15 €) are needed, which further increase the costs.

4. Discussion

Our findings reveal that both eDNA methods are successfully recovering and detecting *A. astaci* spores from lotic water systems, but have advantages and drawbacks inherent in each system. Detection rates obtained with the two filtering approaches were comparable to or slightly higher than those of other eDNA applications in running waters (e.g. Thomsen et al., 2012a; Goldberg et al., 2011; Santas et al., 2013).

Previous studies show that the use of water sampling methods with filters of large pore sizes and large water volumes are beneficial for eDNA-based monitoring of rare species due to heterogeneous distribution of eDNA particles (e.g. Goldberg et al., 2011; Jerde et al., 2011; Turner et al., 2014). The application of the DF method in the field and in the laboratory is relatively easy, fast and cost-effective and proved already suitable with water volumes of 5 l in both small (ponds, streams) and large water systems (lakes) with an adequate number of replicates (Strand et al., 2014). We found that a further speed up in field sampling can be achieved by parallelizing the water filtration with two inline filter holders to produce four DF sample replicates in ~20 min, with the replicate number proving sufficient for reliable *A. astaci*

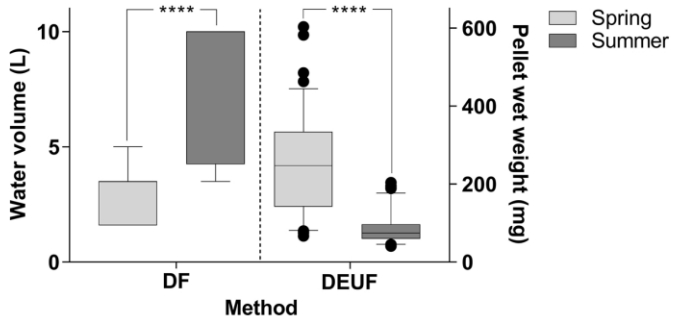

Fig. 6. Comparison of total water volume for DF samples and pellet wet weight for DEUF samples in sampling months May and August.

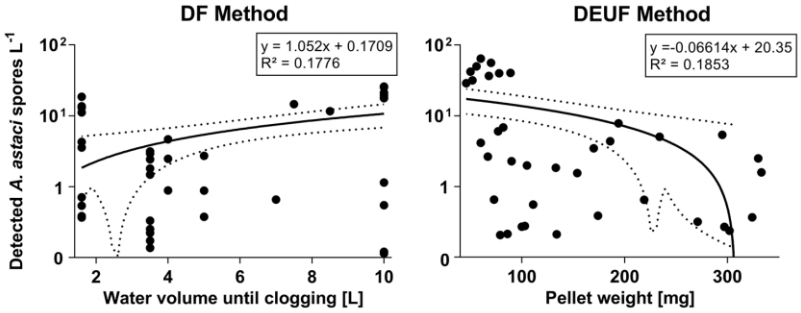

Fig. 7. Detected *A. astaci* spores L^{-1} as function of filtrated water volume until clogging for the DF method (left) or obtained pellet weights of DEUF method (right).

detection in the tested streams. The use of 2 µm glass fibre filters enables to filtrate high water volumes of up to 10 l and equally decreases the clogging potential in turbid waters. Difficulties arise in periods with high precipitation, when filtration capacity of glass fibre filters is significantly reduced due to very fast clogging. As a result, subsamples have to be produced to reach a higher final water filtration volume, which increases costs of filter material and time in the field and during laboratory work.

The major benefit of using the DEUF method is the use of large volumes of water samples (> 100 l), which should increase the likelihood of *A. astaci* detection in large water systems (Wilcox et al., 2013). This finding is supported by our results from summer sampling, when DEUF samples achieved higher spore amounts compared to DF samples. Problematic issues in terms of applicability of the DEUF approach are spore retention in ultrafilters (Smith and Hill, 2009), DNA-loss during centrifugation due to incomplete spore pelleting and strong inhibition

Table 4
Comparison of water filtering procedure with the DF and DEUF method.

Procedure step (location)	DF	DEUF
Preparation (laboratory)	Disinfection: 0.25% PAA inner tubing, 0.5% bleaching solution or Sagrotan® outer tubing and all surfaces **Time needed: 30 min**	
Filter preprocessing (laboratory)	/	Blocking procedure: blocking buffer according to Hill et al. (2005) **Time needed: 10 min per ultrafilter**
Filtration procedure (field)	**6 min/10 L**	Flow rate: 1.600 ml/min **60 min/100 L**
Storage (field)	50 ml screw-cap tubes	Ice box (~4 °C) sealed ultrafilter cartridge
Filter postprocessing (laboratory)	/ /	Elution procedure: elution buffer according to Hill et al. (2005) **Time needed: 10 min per ultrafilter** ~600 ml eluate divided in 12 × 50 ml screw-cap tubes **Time needed: 10 min per ultrafilter**
Storage (laboratory)		−20 °C
Sample processing (laboratory)	/	Pelleting of eluted water sample Transfer to 2 ml reaction tubes, **Time needed: 30 min per extraction round (16 samples)**
DNA extraction		**Time needed: ~5 h**
Inhibition	no or negligible	yes
Dilution	no	yes, 10-fold
Real-time PCR		**Time needed: ~1 h**
Filter costs	0.52 € per GFF	15 € per ultrafilter cartridge (1.25 € per sample)
Problematic issues	Clogging of filters in turbid water condition Low population density	Low spore recovery

in qPCR (Strand et al., 2014), which altogether leads to a significant decrease in sensitivity. Procedures to increase the recovery rates of microbes from ultrafilters have been studied intensively. Treatments of ultrafilters with calf-serum or sodium polyphosphate prior to filtration and ultrafilter elution were found to improve the recovery of microbial material from the ultrafilter matrix (Hill et al., 2005, 2007; Leskinen and Lim, 2008; Lindquist et al., 2007; Polaczyk et al., 2008; Smith and Hill, 2009). From our findings, we conclude that preparing the ultrafilter matrix with sodium polyphosphate and eluting the material with the elution procedure described in Smith and Hill (2009) seemed to be beneficial for the recovery of *A. astaci* spores. Our results indicate in fact a better performance of the DEUF method in summer compared to the DF method, although we were not conducting spore recovery quantification experiments prior to the field application. Mean recovery rates of DEUF approaches have been found to be greater than 50% (Kearns et al., 2008; Leskinen and Lim, 2008; Leskinen et al., 2009; Smith and Hill, 2009; Mull and Hill, 2012) and should be evaluated for *A. astaci* spore recovery in future studies. Strand et al. (2014) concluded from their study that improving the DNA- and zoospore recovery combined with solving the strong PCR inhibition problems would yield at least a 10-fold increase in sensitivity of the DEUF compared to the DF method.

We conclude from our results, that the time period when surveys are carried out as well as the water filtering method used has to be chosen carefully to increase the likelihood of *A. astaci* detection in natural water bodies. Season-dependent water turbidity considerably affected the *A. astaci* detection probability of both eDNA methods. In the study period 2013, precipitation was high in spring and high water turbidity was observed in all tested streams. This is reflected by the significantly higher pellet weights for the DEUF method and significantly lower water volumes for the DF samples in spring, which negatively affect the *A. astaci* spore detection of both water sampling approaches. The observed water volumes for DF samples and pellet weights for DEUF samples act in reverse ways, since high water turbidity affects the filtration capacity of both eDNA methods differently. For the DF method a high particle freight leads to fast clogging and therefore lower amounts of water can be filtrated through the glass fibre filters. For the DEUF method high amounts of suspended matter are captured in the hollow fibre matrix, which leads to increased pellet weights after elution. The increased particle matter seems to impair spore recovery from the filter cartridge, perhaps due to clogging or adverse particle-to-spore ratio, which reduces *A. astaci* spore release from the hollow fibre matrix. Since higher *A. astaci* detection probabilities were obtained with increasing water volume for DF samples and decreasing pellet weights for DEUF samples, we generally recommend to conduct eDNA field surveys when the load of suspended matter in lotic water courses is low, such as periods with low precipitation.

Turner et al. (2014) pointed out that eDNA capturing methods relying on very small particle size cut-offs as used in precipitation, centrifugation and ultrafiltration approaches seem to be generally inferior to filtration methods. With the data gained in our study we can partly confirm this general assumption, since the DF method was performing significantly better in *A. astaci* detection in spring despite the lower filtrated water volumes. Presumably, the poorer performance of the DEUF method is the result of the higher pellet weights and the increased co-extraction of inhibiting substances in the eluate, which was negatively affecting the detection success. Still, the ultrafiltration-based DEUF method detected significantly more *A. astaci* spores in the summer season and was generally more sensitive in detecting *A. astaci* spores. In 89% of cases when only one eDNA method showed a clear positive result (above LOQ of 50 PFU as described by Vrålstad et al., 2009), it was the DEUF method. This ratio indicates that filtration of high-volume water samples with a small size cut-off can be more beneficial for *A. astaci* detection in running waters, especially when the population density of infected carrier crayfish and/or pathogen levels in the population are low.

As both filtration techniques detected significantly more *A. astaci* spores in the summer season, our results are in line with findings in former studies, in which *A. astaci* spore release was found to be modulated by water temperature, when significantly more spores are released from infected carrier crayfish at high water temperatures due to higher moulting rates (Strand et al., 2012; Svoboda et al., 2013; Wittwer et al., 2017).

5. Conclusion

Both eDNA methods yielded high *A. astaci* detection rates and constitute useful tools for eDNA monitoring of *A. astaci* in large water systems. The observed detection rates and pathogen levels of both water sampling strategies were affected by water turbidity, resulting in different relative performance of the methods in spring and summer. While the DEUF method appears to be slightly more sensitive in *A. astaci* detection, its application is more laborious, making the use of this method more profitable for site- specific investigations especially in areas with very low carrier crayfish population densities and low pathogen levels – e.g. in early invasion stages. Due to the fast, cost-effective and easy-to-apply handling in the field and the laboratory and the very robust quantification results of DF samples, the DF method is the more straightforward application for *A. astaci* detection and can be applied easily in running waters for large-scale screenings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.limno.2018.03.001.

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Publication II – Supplementary Material

Supplement Table 1: Coordinates of study sites.

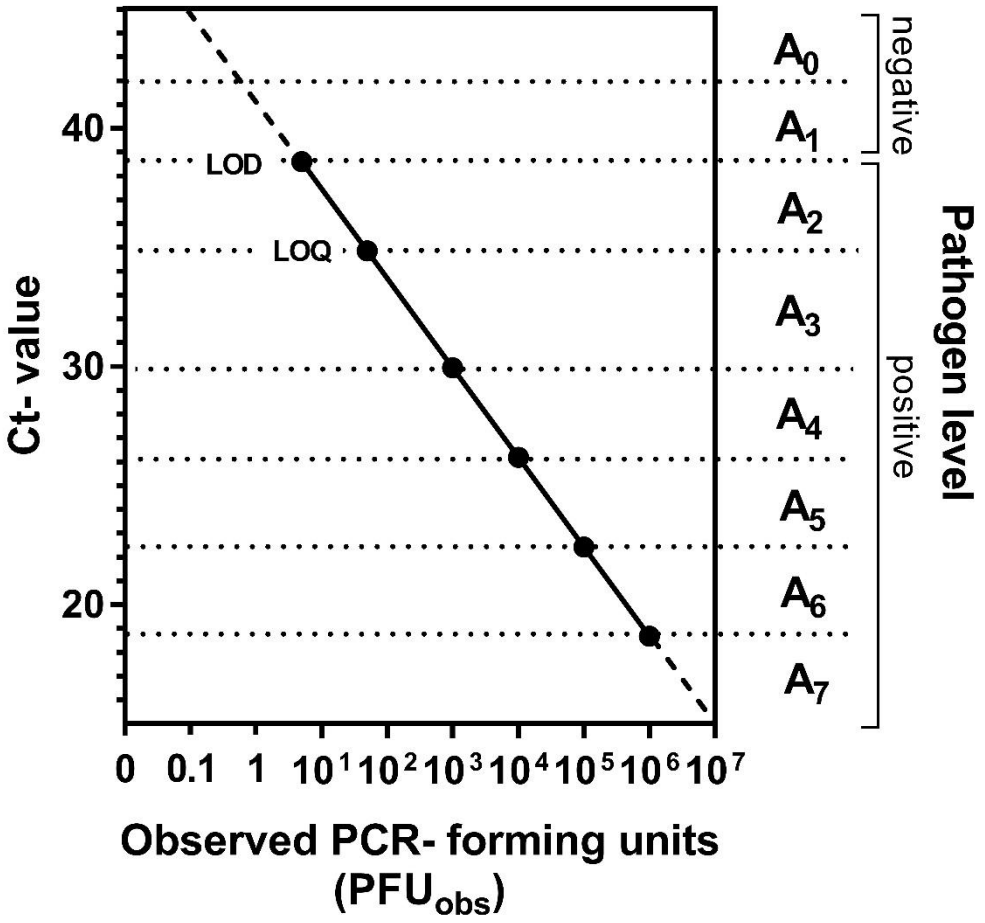
Site	Sampling area		
	Ulmbach	**Lütter**	**Weschnitz**
A	N50.61887 E8.19237	N50.48746 E9.89498	N49.63963 E8.75975
B	N50.60990 E8.25585	N50.49438 E9.88392	N49.62818 E8.76242
C	N50.59653 E8.28078	N50.48924 E9.87235	N49.64061 E8.76776
D	N50.58123 E8.30114	N50.49102 E9.85442	N49.61138 E8.75218
E	N50.57154 E8.29413	N50.49055 E9.83887	N49.60482 E8.74276
F	N50.56154 E8.29210	N50.48424 E9.81823	N49.58919 E8.73090
G	N50.55025 E8.29632	N50.48141 E9.80131	N49.57849 E8.71923
H	N50.53248 E8.30900	N50.47994 E9.76901	N49.56090 E8.69519
Sampling period	May and August 2013		

Supplement Table 2: Filtered water volumes (in L) achieved with DF method.

Location	**Season**	**DF method** **Water volume [L]**							
	Site	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
Ulmbach	spring	4	4	5	4	1.6	1.6	1.6	1.6
	summer	5	4	8.5	10	10	10	10	7.5
Lütter	spring	1.6	1.6	1.6	1.6	5	1.6	1.6	1.6
	summer	10	10	10	10	10	10	10	10
Weschnit	spring	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
	summer	3.5	3.5	10	5	3.5	3.5	7	3.5

Supplement Table 3: Averaged pellet weights (in mg) achieved with DEUF method. Pellets of 50 ml DEUF subsamples representing the spore content in approximately ~8.333 L of filtered water.

Location	Season	DEUF method Pellet weight [mg]							
	Site	A	B	C	D	E	F	G	H
Ulmbach	spring	278	219	420	333	234	194	330	295
	summer	66	82	89	50	70	60	52	46
Lütter	spring	126	154	105	174	82	103	86	134
	summer	78	68	56	60	79	100	109	63
Weschnit	spring	409	355	269	324	271	302	297	379
	summer	67	77	73	186	170	133	111	90



Supplement Figure 1: Pathogen levels are log-ranked semi-quantitative categories based on PCR- forming units (PFU_{obs}) according to Vrålstad et al. (2009). Mean cycle threshold (Ct) values of the master standard curve are plotted against observed mean PFU values. Pathogen levels A_0 (no detection) and A_1 (below the limit of detection/LOD of ≤ 5 PFU_{obs}) are both considered negative. The pathogen level A_2 is ranked between $5 \leq PFU_{obs} < 50$, which is also the limit of quantification/LOQ. Subsequent pathogen levels are as follows: A_3 between $50 \leq PFU_{obs} < 10^3$, A_4 between $10^3 \leq PFU_{obs} < 10^4$, A_5 between $10^4 \leq PFU_{obs} < 10^5$, A_6 between $10^5 \leq PFU_{obs} < 10^6$ and A_7 with $10^6 \leq PFU_{obs}$.

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Declaration on the contributions of authors

What has the PhD candidate contributed, and what have the coauthors contributed?

(1) Development and planning
Coauthor MT: 70%
PhD candidate and coauthors SS and CN each 10%

(2) Implementation of the respective studies and experiments
PhD candidate: 100% – field work (collecting water and tissue samples), molecular analysis

(3) Creation of the data collection and figures
PhD candidate: 100% – created database, created figures

(4) Analysis and interpretation of the data
PhD candidate: 80% – analysis and interpretation of field and molecular data
Coauthor SS 10% – contributed to data analysis and interpretation
Coauthors CN and MT each 5% – contributed to data analysis and interpretation

(5) Writing the manuscript
PhD candidate: 85%
Coauthor CN: 10%
Coauthor SS: 5%

Date/place: _____ / Frankfurt am Main, Germany

Signature PhD candidate: _____

Affirmative confirmation of the above information:

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ORIGINAL PAPER

eDNA-based crayfish plague detection as practical tool for biomonitoring and risk assessment of *A. astaci*-positive crayfish populations

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Abstract While environmental DNA (eDNA) approaches have the potential to revolutionize biodiversity research and monitoring, most aquatic biomonitoring and management programs still rely on conventional monitoring methodologies. Here we evaluated the suitability and robustness of an eDNA-based approach for the detection of the crayfish plague agent *Aphanomyces astaci* and assessed the capacity of this new method as practical tool for bioassessments in freshwater. The approach was examined in three case studies: case study A tested its use as biomonitoring tool to determine the range and density of *A. astaci*- infected crayfish populations in three different water systems. Case study B focussed on the identification of possible *A. astaci* infection sources, here an aquafarm. For case study C we assessed a migration front of infected crayfish near a native crayfish area. The eDNA-based detection allowed to infer local patterns and distribution limits of crayfish plague occurrence. Spore estimates in eDNA samples correlated significantly with catch per unit effort values and pathogen loads of captured *A. astaci*- positive crayfish obtained from trapping in running waters. We also showed that spore concentrations are detectable up to

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three kilometres downstream from hot spot areas of infected crayfish. By identifying a possible *A. astaci* entry point and migration front eDNA proved suitable for the detection of *A. astaci* spores at low population densities and/or pathogen levels of infected crayfish. The study provides conclusive evidence for the suitability of the eDNA approach as a tool for risk assessment and large-scale monitoring of *A. astaci* for a wide range of practical conservation issues of indigenous crayfish species.

Keywords Environmental DNA · *Aphanomyces astaci* · *Pacifastacus leniusculus* · Density dependence · Biomonitoring · Risk management

Introduction

The use of environmental DNA (eDNA) methods promises to revolutionize biodiversity research and monitoring (Bohmann et al. 2014; Goldberg et al. 2011; Taberlet et al. 2012a; Thomsen and Willerslev 2015). In recent years numerous eDNA studies have shown high suitability and robustness in single species (rare/endangered species: e.g. Thomsen et al. 2012a; Goldberg et al. 2011; invasive species: e.g. Adrian-Kalchhauser and Burkhardt-Holm 2016; Dougherty et al. 2016; Ficetola et al. 2008; Jerde et al. 2011; pathogens: e.g. Huver et al. 2015; Wittwer et al. 2018a) and community level assessments (e.g. Bálint et al. 2018; Taberlet et al. 2012b; Thomsen et al. 2012b; Valentini et al. 2016).

Despite of the advances achieved in eDNA research during the past decade, the majority of current aquatic biomonitoring and management programs still rely on conventional, often destructive monitoring tools. Previous eDNA studies showed similar or higher detection probabilities of target species achieved with eDNA-based methods compared to traditional survey methods (e.g. Dejean et al. 2012; Smart et al. 2015; Tréguier et al. 2014; Wittwer et al. 2018a). Only rarely, however, these new eDNA tools have been used in large-scale monitoring attempts (e.g. Biggs et al. 2015). Many open questions exist regarding factors that may hamper standardization of eDNA methodologies in aquatic environments (Barnes and Turner 2016; Bohmann et al. 2014; Goldberg et al. 2016; Rees et al. 2014, 2015; Roussel et al. 2015), e.g. sediment type (Pietramellara et al. 2009), water chemistry (Barnes et al. 2014; Strickler et al. 2015), flow intensities and downstream transport (Deiner and Altermatt 2014; Fukumoto et al. 2015; Jane et al. 2015; Pilliod et al. 2014; Rice et al. 2018; Wilcox et al. 2016), dilution and degradation processes (Dejean et al. 2011; Roussel et al. 2015; Strickler et al. 2015) or the sampling and extraction protocols (Deiner et al. 2015; Geerts et al. 2018; Minamoto et al. 2016; Wittwer et al. 2018b). These issues may lead to low detection rates of target organisms (Roussel et al. 2015), which could impede the transfer of eDNA applications into official monitoring programs. Thus we aimed to test if eDNA-based single-species detection at its current state can be applied to answer crucial questions in applied water management and species conservation. For this we chose a relevant example, namely the crayfish plague agent *Aphanomyces astaci* (Schikora 1906), which is regarded as a major threat to European crayfish biodiversity (Taylor 2002). *A. astaci* is a waterborne oomycete (Beakes et al. 2014) causing the crayfish plague among susceptible crayfish species via motile zoospores (Alderman and Polglase 1988). Zoospores are continuously released from infected crayfish (Strand et al. 2012) with peak zoospore production prior to or soon after death (Evans and Edgerton 2002). The pathogen was introduced to Europe by the import of North American crayfish species such as the signal crayfish *Pacifastacus leniusculus* (Dana 1852; Crandall 2017a; Unestam 1972) for commercial exploitation in the late 19th century (Alderman and Polglase 1988). North American crayfish species are known carriers of the disease agent and at least partially resistant to *A. astaci* infection due to fast immune reaction which restricts hyphal growth (Unestam and Nylund 1972; Unestam and Weiss 1970). Native crayfish species largely fail to constrain *A. astaci* growth and thus die soon after infection (Unestam and Weiss 1970). The spread of this pathogen to new water bodies has devastating effects on native crayfish species such as the noble crayfish *Astacus astacus* (Linnaeus 1758; Crandall 2017b), which is today considered as "vulnerable" species on the IUCN Red list (Edsman et al. 2010). The widespread occurrence of invasive crayfish species in European water bodies (Kouba et al. 2014) implies a spatially extended co-existence of *A. astaci*, but no detailed *A. astaci* distribution map exists to date (DAISIE 2008). *A. astaci* spreads via migrating

infected crayfish (e.g. Bubb et al. 2004), contaminated fishing equipment (Alderman 1996) or predatory species (e.g. eels; Evans and Edgerton 2002). The continuing dispersal of *A. astaci* and invasive crayfish species hampers the conservation of relict populations of native crayfish species and raises concerns over their future survival in European water bodies (Holdich et al. 2009).

To be suitable for large-scale monitoring approaches, eDNA results must reflect real population densities, in this study of *A. astaci*-infected crayfish populations, to allow comparisons with conventional survey methods such as trapping assessments. Numerous eDNA studies provide good estimates of population density (e.g. Thomsen et al. 2012a; Dunn et al. 2017), abundance (e.g. Lodge et al. 2012; Pilliod et al. 2013) and biomass (e.g. Takahara et al. 2012), suggesting that a correlation between eDNA-based spore estimates and abundance of infected crayfish is at least plausible. eDNA methods also have to work reliable in all water bodies under different environmental circumstances. Especially the detection in running waters is of particular importance, as they are the major distributors for crayfish plague dispersal across landscapes (Svoboda et al. 2017). High assay specificity and sensitivity of eDNA methods are necessary to detect target species in very low population densities (Rees et al. 2014), e.g. during the early phase of invasion. Efficient implementation of eDNA methods will extend the knowledge on potential *A. astaci* infection sources and invasion fronts close to Native Crayfish Areas (NCAs, Taugbøl and Skurdal 1999) or "ark sites" (Peay 2009; Holdich et al. 2009), which are of particular importance for the conservation of native crayfish species.

We used an existing robust eDNA marker set for *A. astaci* detection (Strand et al. 2014; Wittwer et al. 2018a, b) in the course of three case studies of crayfish plague assessments in central Germany (Figs. 1, 2, 3, 4, up) to answer the following questions:

- *Case study A—density dependence* Can we estimate the range and density of infected invasive crayfish based on *A. astaci* spore estimates in the course of different water systems? Do spore concentrations estimated from eDNA results correlate with population densities or pathogen loads of infected crayfish populations obtained from conventional trapping approaches?
- *Case study B—aquafarm surveillance* Do aquafarms serve as entry points for *A. astaci* spores and/or infected crayfish?
- *Case study C—NCA management* Do eDNA-derived spore estimates allow to locate a migration front of *A. astaci*- positive crayfish near a NCA?

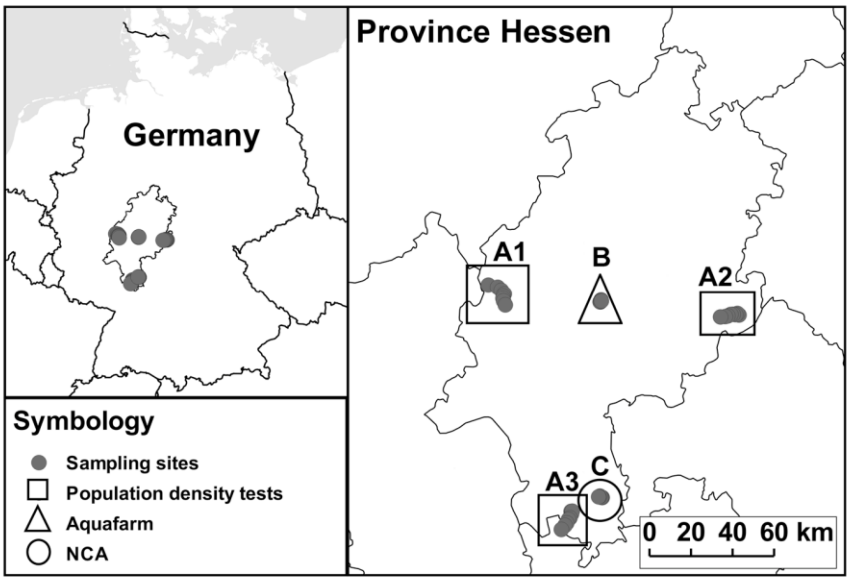

Fig. 1 Sampling areas targeted in this study were located in Germany in the federal state Hessen. Samples for case study A were taken in the streams Ulmbach (A1), Lütter (A2) and Weschnitz (A3), for case study B in an aquafarm in the Lauter-Wetter water system and for case study C near a NCA of *A. astacus* in the Mümling water course. Exact coordinates and the distance between the individual sampling sites are listed in Supplement Table 1

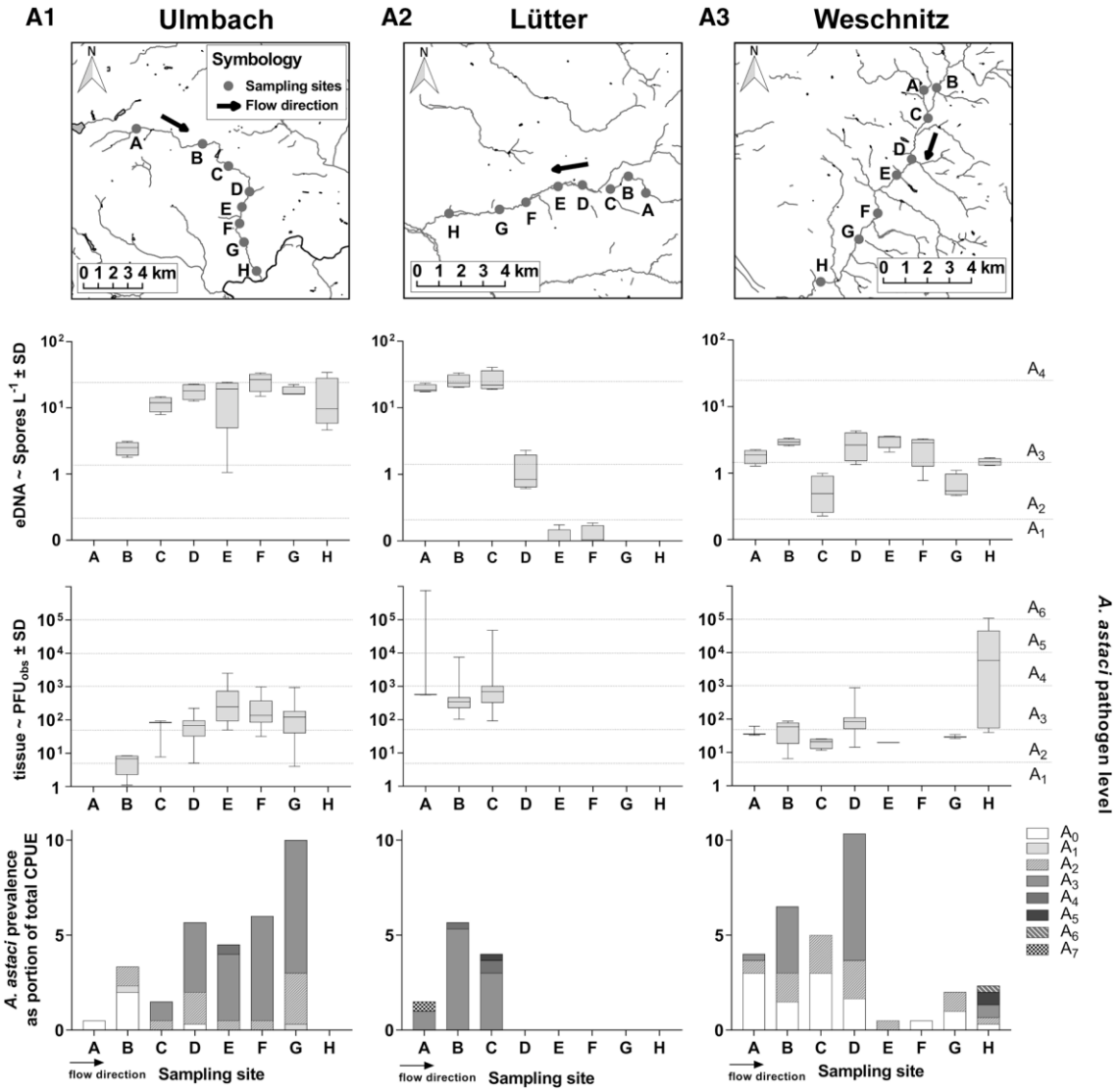

Fig. 2 Case study A. Fine-scale maps and results from the three streams Ulmbach (A1), Lütter (A2) and Weschnitz (A3) comparing mean *A. astaci* spore estimate (spores $L^{-1} \pm$ SD) in eDNA samples (N = 4), observed *A. astaci* PCR forming units ($PFU_{obs} \pm$ SD) in crayfish tissue samples (N = captured at specific site according to Table 1) and *A. astaci* prevalence as portion of total Catch Per Unit Effort (CPUE; N = see above, Table 1) and corresponding pathogen levels

Materials and methods

Study sites and crayfish trapping

Five sampling areas were chosen in the federal state of Hessen in Central Germany to conduct different case studies on population density dependence (A) and risk assessment in an aquafarm (B) and a NCA (C, Fig. 1).

Case study A investigates the effect of population density of infected crayfish on the *A. astaci* spore estimates in eDNA samples (Fig. 2, A1–A3, up). For this eight independent sampling sites were selected in three streams (A1: Ulmbach, A2: Lütter and A3: Weschnitz; exact coordinates and distances are given in Supplement Table 1), where *P. leniusculus* is known to occur in well-established and *A. astaci*-

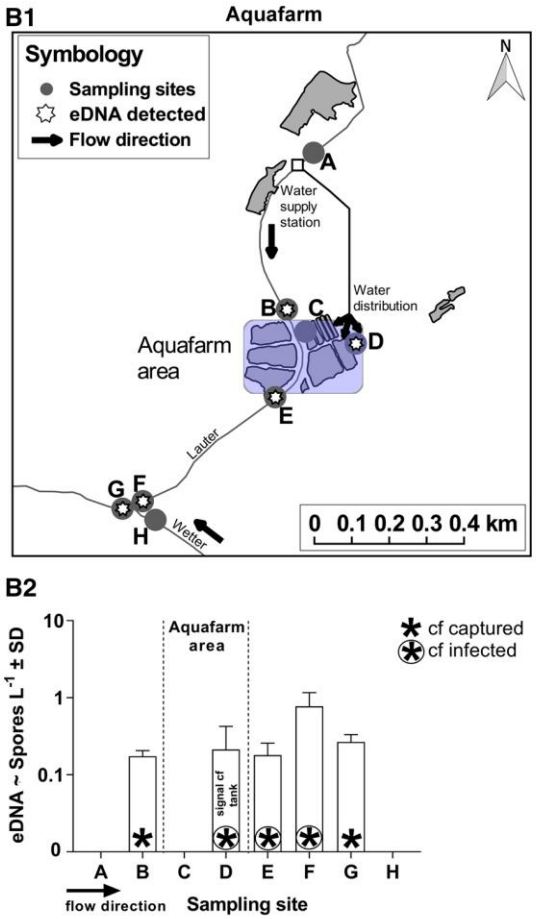

Fig. 3 Case study B. Fine-scale map (B1) and results of eDNA sampling (B2) of an aquafarm. Asterisks indicate, that crayfish (cf) were captured (see Table 1), circled asterisks indicate *A. astaci*-infected crayfish

positive populations. Parts of eDNA and tissue sample data were previously published in Wittwer et al. (2018a, b) as indicated in corresponding tables. To evaluate the potential of eDNA as risk assessment tool at very low population densities and/or pathogen levels of infected crayfish species, we examined two different scenarios. For case study B we tested a potential invasion pathway outgoing from an aquafarm, which could serve as entry point for *A. astaci* spores or infected crayfish (Fig. 3, B1). For this we collected eDNA samples at eight sampling sites in aquafarm ponds and along the stream Lauter. In case study C an upstream migration pathway of an infected *P. leniusculus* population near ponds inhabited by

Table 1 Number of crayfish trapped per site in case studies A and B with trap numbers in parentheses. Captured individuals from the stream Ulmbach at sites B, D, G and H were previously published in Wittwer et al. (2018a)

Site	Sampling area			
	Case study A			Case study B
	Ulmbach	Lütter	Weschnitz	Aquafarm
A	1 (2)	3 (2)	12 (3)	0 (3)
B	10 (3)	17 (3)	13 (2)	1 (2)
C	3 (2)	12 (3)	10 (2)	0 (1)
D	17 (3)	0 (2)	31 (3)	5 (1)
E	9 (2)	0 (2)	1 (2)	1 (4)
F	12 (2)	0 (3)	1 (2)	1 (3)
G	30 (3)	0 (3)	6 (3)	1 (3)
H	0 (3)	0 (2)	7 (3)	0 (3)
Total	82 (20)	32 (20)	81 (20)	9 (20)

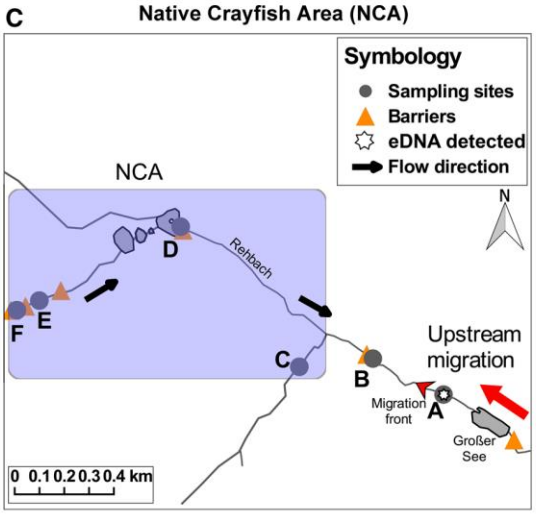

Fig. 4 Case study C with fine-scale map and results of eDNA sampling from a NCA

native *A. astacus* populations serving as NCAs was surveyed at six sampling sites (Fig. 4). Sampling took place in August 2013 for Ulmbach, Lütter and Weschnitz, in August 2014 for the aquaculture and its nearby water course Lauter and in September 2014 for the NCA and its pond system in the Mümling water course. Crayfish were captured with traps of type "Pirat"(Engel-Netze GmbH & Co.KG, Germany).

Table 2 *A. astaci* detection in eDNA and tissue samples (in % of positive samples) of case study A. *A. astaci* PFU (PCR forming units) as determined by qPCR analysis according to Vrålstad et al. (2009) are ranked into pathogen levels[1]. Tissue and eDNA results of the stream Ulmbach at sites B, D, G and H were previously published in Wittwer et al. (2018a) and eDNA results were previously published in Wittwer et al. (2018b)

Location	No.	eDNA					*A. astaci* detected (%)	No.	Tissue								*A. astaci* detected (%)
		Pathogen level[1]							Pathogen level[1]								
		Negative		Positive					Negative		Positive						
		A0	A1	A2	A3	A4			A0	A1	A2	A3	A4	A5	A6	A7	
Ulmbach	32	4	0	1	12	15	88	82	8	2	19	53	1	0	0	0	88
Lütter	32	8	4	5	3	12	63	32	0	0	0	27	3	1	0	1	100
Weschnitz	32	0	0	15	17	0	100	81	28	0	20	30	0	2	1	0	65
Total	96	12	4	21	32	27	83	195	36	2	39	110	4	3	1	1	84

[1]Pathogen levels A_0 (no detection) and A_1 ($\leq$ 5 PFU_{obs}; below LOD) are both considered negative; positive pathogen level ranks are A_2: $5 \leq PFU_{obs} < 50$ (LOQ), A_3: $50 \leq PFU_{obs} < 10^3$, A_4:$10^3 \leq PFU_{obs} < 10^4$, A_5: $10^4 \leq PFU_{obs} < 10^5$, A_6: $10^5 \leq PFU_{obs} < 10^6$, A_7: $10^6 \leq PFU_{obs}$

Traps baited with dog treat were deployed at each sampling site of Ulmbach, Lütter and Weschnitz and in the Lauter system covering the aquafarm area for one night. Carapax size and sex of captured individuals were recorded (Supplement Table 2). All captured crayfish (Table 1) were taken to the laboratory on ice and frozen at − 20 °C until further analysis. Catch per unit effort (CPUE) values were determined by dividing the total number of captured crayfish with the number of traps. Permission for crayfish and water sampling was obtained from the responsible aquafarm owners and conservation authorities (Table 2).

eDNA sampling

An eDNA procedure based on depth filtration (Agersnap et al. 2017; Strand et al. 2014; Wittwer et al. 2018a) was performed to obtain four independent eDNA samples at each sampling site. Glass fibre filters (AP25; Ø 47 mm; 2 µm pore size; Millipore, Merck KGaA, Germany) were used to filter water samples directly in the field with a peristaltic pump apparatus (Masterflex, Cole-Palmer Instrument Company, LLC, USA). Filters were placed on clean in-line filter holders (Millipore, Merck KGaA, Germany) with sterile forceps and disposable glows. Initially and between the individual sites, the tubing and the filter holders were cleaned with disinfectant (inside tubing: peracetic acid; outside tubing and equipment: isopropanolic/ethanolic disinfectant; filter holders: 20% bleach solution and ethanol). Prior to filtration at a sampling site, water was initially pumped through the apparatus to remove whirled up sediment particles and residual disinfectant from the system. Filtered water volume ranged between 1.6 and 10 L depending on water turbidity (Supplement Table 3). When water was sampled from highly turbid ponds, water was initially pumped with a bilge pump system from the bottom following the description in Wittwer et al. (2018a). If a water volume of 1.6 L was not reached with one glass fibre filter in highly turbid waters at certain sites (mainly ponds, but also during rain in lotic water courses), subsamples were produced to increase total water filtration volume according to Wittwer et al. (2018a). After filtration the filters were transferred to a sterile falcon tube, stored on ice during transport and at − 20 °C in the laboratory until further processing. After each sampling event and between sampling areas the complete equipment was thoroughly cleaned and disinfected with 0.25% peracetic acid, isopropanolic/ethanolic disinfectant and 20% bleach solution.

DNA extraction from tissue and eDNA samples

DNA was extracted from tissue of all signal crayfish captured in this study with CTAB extraction according to Vrålstad et al. (2009) from parts of soft cuticle, tail fan and a walking leg. eDNA samples were extracted with an optimized CTAB protocol for complete glass

fibre filter extraction according to Wittwer et al. (2018a). For this 4 ml CTAB Buffer (20 g L^{-1} CTAB, 1.4 M NaCl, 0.1 M Tris–HCl, 20 mM Na_2EDTA) was added to a filter and a subsequent freezing step for 30 min at − 80 °C was conducted for cell lysis. After thawing at 65 °C, 40 µl proteinase K (20 mg ml^{-1}) was added and samples were incubated at 65 °C for 1 h. 3 ml chloroform was added and the tubes were shaken manually for approx. 15 s. After centrifugation (15 min, 3.005 *g*), the upper phase (∼ 1500 µl) was transferred to a 5 ml reaction tube (Eppendorf AG, Hamburg, Germany). The subsequent extraction procedure followed the description in Wittwer et al. (2018a) in using 1000 µl chloroform, 1200 µl supernatant, 1800 µl isopropanol, 600 µl EtOH (70%). Depending on the number of subsamples produced at certain sampling sites, DNA pellets were resuspended in 100 µl TE buffer (pH 8.0) for one GFF sample/sample, whereas for two subsamples 2 × 50 µl and for four subsamples 4 × 25 µl TE buffer was added to each subsample pellet with subsequent pooling of DNA extracts. An extraction blank control consisting of 4 ml CTAB buffer was included in each extraction run. All eDNA extractions were conducted in a low-DNA laboratory under strict working conditions to avoid cross-contamination.

qPCR and data analysis

An *A. astaci*- specific qPCR assay according to Vrålstad et al. (2009) was used to detect and quantify the crayfish plague agent in water and tissue samples on a TOptical Gradient 96 qPCR system (Biometra, Analytik Jena, Göttingen). The qPCR protocol was slightly changed in using TaqMan® Environmental Master Mix 2.0 and temperature/time conditions with 62 °C annealing for 30 s according to Strand et al. (2014). Samples were run with triplicates (case study A) or quadruple (case studies B and C) with undiluted eDNA samples and tenfold diluted tissue samples due to massive inhibition in tissue samples and minor inhibitory effects in eDNA samples (Wittwer et al. 2018a). No template (NTC) and negative extraction controls were run on all 96-well plates and did not amplify any target on each qPCR run in this study. On each plate four standard concentrations of extracted *A. astaci* tissue were run in duplicate as positive controls and for master standard curve construction. The software qPCRSoft 3.1 (Biometra, Analytik Jena, Göttingen) was used for the calculation of Ct values. PFU (PCR forming unit) concentration per sample was calculated from averaged Ct values with the use of master standard curves. For case study A (Ulmbach, Lütter, Weschnitz) standards of a complete dataset obtained during previous studies (Wittwer et al. 2018a, b) were used to construct a master standard curve (formula: $y = (-1.633)*\ln(x) + 41.232$; $r^2 = 0.99$; slope = − 3.7598; efficiency 85%). For case studies B (aquafarm) and C (NCA) a new master standard curve was generated (formula: $y = (-1{,}617004)*\ln(x) + 40{,}024986$; $r^2 = 0{,}99$; slope = − 3,7233; efficiency 86%) from fresh *A. astaci* mycelium. For eDNA samples, averaged PFU values of undiluted water samples were used to calculate spore estimates according to Strand et al. (2014). The mean PFU value was multiplied by 20 (1/20 of DNA extract used for qPCR) and divided by 138 (estimated PFU value per spore; Strand et al. 2011). Obtained spore estimates were divided by the total amount of filtered water per sample (spores L^{-1}; Supplement Table 3). Mean PFU values of crayfish tissue samples were multiplied by 10 to account for the dilution factor. Samples were regarded *A. astaci*-positive when the mean of all qPCR replicates of one sample exceeded the assay specific limit of detection (LOD) of 5 PFU according to Vrålstad et al. (2009). Pathogen levels correspond to agent levels as introduced by Vrålstad et al. (2009) according to Wittwer et al. (2018a). These levels are based on the observed PFU values (PFU_{obs}) and are used as semi-quantitative categories.

Statistics

The software GraphPad Prism version 7.01 (GraphPad Software Inc., La Jolla California, USA, 2016) was used for statistical data analysis and graphical visualization. One-way ANOVA followed by Bonferroni's multiple comparison tests were performed for eDNA and tissue samples to compare mean spore estimates/PFU_{obs} values across the sampling sites of each individual stream. For evaluating the dependence on population density and pathogen loads in infected crayfish populations, a linear regression was performed on the spore estimates versus the observed CPUE values and a nonlinear regression on the estimated *A. astaci* spore concentrations in eDNA samples versus observed PFU values in tissue samples.

Additional linear regressions were performed on the detection probability and for sensitivity testing on the obtained pathogen levels of both methods of all sampling sites of the test streams. Statistical significance was set at $\alpha = 0.05$.

Results

Case study A: population density tests

A. astaci was detected in all tested streams, but distribution ranges, estimated spore concentrations, prevalences in the crayfish populations and pathogen loads of *A. astaci*- positive crayfish differed among streams (Fig. 2, Table 1). In the Ulmbach stream (Fig. 2, A1) *A. astaci* was detected with eDNA and tissue samples in most parts of the water course with both methods reaching a detection success of 88%. In the stream Lütter (Fig. 2, A2) a distribution hot spot of signal crayfish was observed in the first three sampling sites A–C with a total of 32 captured individuals. All crayfish captured at these sites showed *A. astaci* infection and observed pathogen levels ranged between A_3 and A_7. Correspondingly, estimated *A. astaci* spore concentrations in eDNA samples were significantly higher in the first three sites compared to all following sites downstream ($p < 0.0001$; one-way ANOVA). In the stream Weschnitz (Fig. 2, A3) *A. astaci* prevalence in eDNA and tissue samples was rather low, yet similar CPUE values were obtained compared to the other test streams. *A. astaci* was detected from eDNA samples at all sites along the stream (100%), while 65% (53 of 81) of captured individuals were *A. astaci*-positive. Obtained CPUE values were positively correlated with *A. astaci* spore estimates in eDNA samples (Fig. 5, part 1; linear regression: $p < 0.05$), but no correlation was found between CPUE and observed PFU values in tissue samples. Nonlinear regression revealed that spore estimates in eDNA samples seemed to correlate with PFU value in tissue samples up to a certain point. Estimated *A. astaci* spore concentrations passed into a plateau at $\sim$ 20 spores L^{-1} (high spore concentration; Strand et al. 2014) and only the PFU level increased further (Fig. 5, part 2). Comparing the achieved detectability (Fig. 5, part 3) and pathogen levels (Fig. 5, part 4), *A. astaci* detection was more likely with the eDNA method (linear regression; $p < 0.01$), which generally seemed to be more sensitive (linear regression; $p < 0.01$) in detecting *A. astaci* in the three test streams. In certain cases—e.g. high pathogen loads in individual infected crayfish—the conventional tissue-based method was able to detect far greater pathogen levels.

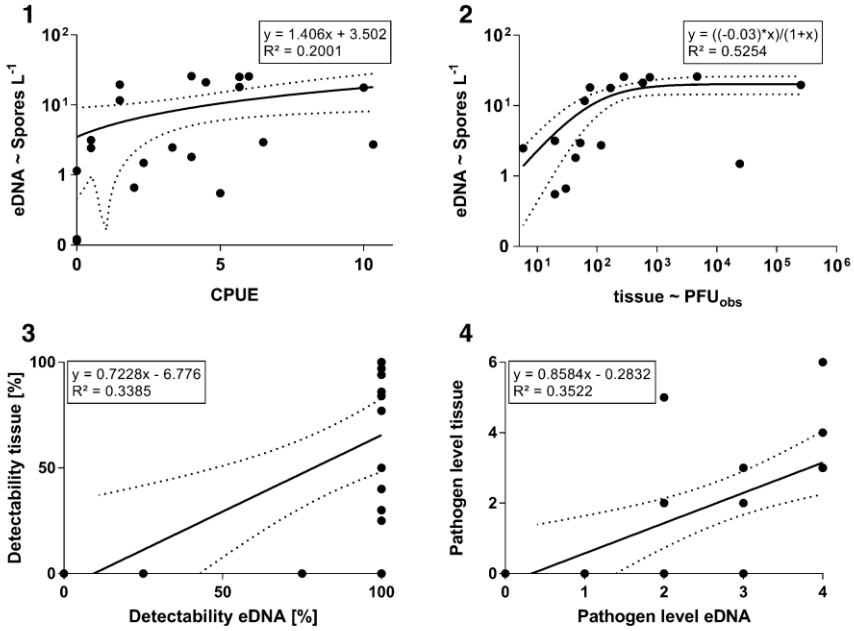

Fig. 5 Comparison of estimated *A. astaci* spores L^{-1} in eDNA samples versus observed CPUE values (1) and observed PFU values in tissue samples (2). *A. astaci* detectability (3) and achieved pathogen levels (4) were compared between the eDNA-based and the tissue-based method. The lines show linear regression with 95% CI for (1) with $R^2 = 0.2001$ ($p < 0.05$), (3) with $R^2 = 0.3385$ ($p < 0.01$) and (4) with $R^2 = 0.3522$ ($p < 0.01$) and nonlinear regression for (2) with $R^2 = 0.5254$

Case study B: aquafarm surveillance

A. astaci was detected in eDNA samples at site B directly upstream the aquaculture with a spore concentration of $\sim$ 0.17 spores L^{-1} (Fig. 3). At sites downstream from the aquafarm, varying spore estimates were observed in all independent samples of sites E ($\sim$ 0.18 spores L^{-1}) and F ($\sim$ 0.78 spores L^{-1}). Site G is located downstream of the inflow of the reference stream Wetter, whose water flow possibly diluted the eDNA signal to an *A. astaci* spore estimate of $\sim$ 0.26 spores L^{-1}. Near the water supply station (site A), in an aquafarm pond (site C) and in the reference stream Wetter (site H), no spores were detected. In the signal crayfish pond (site D), an *A. astaci* spore estimate of $\sim$ 0.21 spores L^{-1} was observed. Other aquaculture ponds were tested as well, but led to no evidence of *A. astaci* occurrence in eDNA samples (data not shown). With trapping four signal crayfish were captured along the stream Lauter downstream from the aquafarm (between sites B to G), with two individuals exhibiting no infection (A_0) and two signal crayfish with a pathogen level of A_3. Five signal crayfish captured in the signal crayfish tank showed differing *A. astaci* infection status ($3 \times A_0$, $1 \times A_2$, $1 \times A_3$).

Case study C: NCA management

A. astaci was detected in water samples at site A with a spore estimate of $\sim$ 0.85 spores L^{-1} near a NCA of native noble crayfish (Fig. 4). eDNA samples taken from upstream sampling sites (B to F) with known occurrence of native crayfish species were tested consistently negative for *A. astaci* (A_0; data not shown).

Discussion

In this study we show that eDNA-based *A. astaci* detection is a suitable tool for bioassessments in freshwater due to its highly consistent detection patterns across a broad range of applied management issues. This outcome is in line with previous findings using the same methodology (Agersnap et al. 2017; Strand et al. 2014; Wittwer et al. 2018b), but typically focused on individual and specific tasks. We show that even in extreme cases with low population density (< 0.5 CPUE) of *A. astaci*- infected crayfish—e.g. during invasion or upstream migration—this method is able to reveal measurable *A. astaci* spore concentrations in running waters. Strand et al. (2014) provided the basis of future crayfish plague monitoring in large natural water systems via eDNA by demonstrating that high *A. astaci* prevalences in crayfish populations and high pathogen loads in crayfish tissue are associated with high spore estimates in eDNA samples. Here we detail these findings and show that in streams inhabited by well-established *A. astaci*-positive signal crayfish populations (case study A) estimated *A. astaci* spore concentrations detected via eDNA are correlating with population densities (measured with the population size estimator CPUE) and pathogen load obtained from conventional trapping approaches. These largely consistent patterns allow to depict the distribution zone of infected crayfish in a stream based on eDNA from water samples. eDNA-based detection was also able to reveal distribution limits of infected crayfish populations comparable to CPUE measurements. For the stream Ulmbach (A1) an upper limit of *A. astaci*-positive signal crayfish distribution was shown with eDNA and trapping, when no spores and only one *A. astaci*- free crayfish were detected at the first sampling site. We presume that site A was at the front line of the distribution of infected crayfish and further migration was successfully prevented by a weir. In the stream Lütter (A2), a hot spot area of infected signal crayfish in the first three sites was found. In this area high spore estimates were observed and captured crayfish exhibited very high pathogen loads. CPUE values and estimated *A. astaci* spore concentrations in eDNA samples in the first three sites were both at its maxima, clearly demonstrating population density dependent factors influencing the detection success for the eDNA method. Moreover, the initially very high spore estimates in eDNA samples in the localized area was steadily decreasing after the hot spot of infected signal crayfish distribution. Interestingly, downstream from site C, no crayfish was captured, but estimated *A. astaci* concentrations were still quantifiable three kilometres downstream of the distribution hot spot of infected signal crayfish (distance between sites C and E). The transport of eDNA to downstream areas is a well-investigated issue, varying from a few to hundreds of metres (Pilliod et al. 2014; Jane et al. 2015) up to several kilometres (Deiner and Altermatt 2014;

Wilcox et al. 2016) distance from the original location of target organisms. For *A. astaci* spores, Strand et al. (2014) assumed a downstream transport from high density areas of infected signal crayfish quite similar to free-floating eDNA molecules. Our observation appears plausible, suggesting that a high density occurrence of infected crayfish can still be measured via eDNA sampling several kilometers downstream. The comparably low *A. astaci* spore estimates and CPUE values of the stream Weschnitz (A3) seem to be the result of a continuing effort to remove invasive crayfish over many years (Hennings, personal comm.). Decreasing the number of infected crayfish thus leads to declining total spore concentrations in eDNA samples.

The eDNA results for risk assessment of aquafarms and NCAs have important implications for the conservation of native crayfish species. For case study B (Fig. 3), our results imply that aquafarm facilities can serve as *A. astaci* entry points to nearby water courses either in terms of water outflow or escaped individuals. eDNA results were negative at the fresh water supply station 500 m upstream of the aquafarm (site A) and varying degrees of *A. astaci* spore estimates were observed at all sites downstream. The observed spore concentrations in the water course are most likely originating from the infected signal crayfish in the stream. Since no "natural" signal crayfish population was known to exist in this area (Dümpelmann, personal comm.), these individuals could be the descendants of escaped crayfish from the signal crayfish pond. It is known that signal crayfish tend to migrate to new habitats, when holding capacity is exceeded (Hogger 1988). Since just a few zoospores are needed to drive a complete native population sensitive for *A. astaci* to extinction (Kozubíková et al. 2008), accurate crayfish and water management strategies in aquafarms are important to contain *A. astaci* transmission to connected waterways. High security standards should be maintained when emptying ponds for cleaning or preparation of new fish stockings, because the outflowing water could carry spores or juveniles to the connected streams and harm all native crayfish species downstream.

For case study C (NCA), eDNA showed correct negative results in ponds inhabited by the noble crayfish. *A. astaci* spore estimates were only observed at the first site upstream of a large lake, which was already known to inhabit signal crayfish (Hennings, personal comm.). We suggest that noble crayfish in the NCA will be afflicted by an upstream migrating signal crayfish in the near future due to their high dispersal rates (Bubb et al. 2004). Generally, the rate of spread of infected crayfish hosts is dependent on population density, flow rates of a given stream and the presence of barriers to crayfish movement (Alderman & Polglase 1988; Evans and Edgerton 2002). In previous studies natural or man-made barriers like waterfalls or weirs and dams were shown to work as obstacle for further migration of infected crayfish (Gherardi et al. 2011; Kozubíková et al. 2008; Taugbøl et al. 1993). Thus restoration of natural water systems in regard to restore river continuity as implied by the EU Water Framework Directive (2000/60/EG) could have deleterious effects. Removing weirs, especially in regions with NCAs, simplifies further spread of infected invasive crayfish species. Leaving some weir structures (Rahel 2013) as obstacles for further migration of *A. astaci*-positive crayfish would be beneficial for the viablity of vulnerable crayfish species in upstream parts (e.g. Light 2003; Kozubíková-Balcarová et al. 2014).

The application of eDNA in aquatic monitoring assessments can have many advantages compared to traditional methods, such as lower working hours in the field (Biggs et al. 2015), higher cost efficiency (depending on the number of investigated sites and taxa, Shepherd et al. 2014; Bálint et al. 2018) or increased detection probabilities (e.g. Smart et al. 2015; Wittwer et al. 2018a). Still, detection via eDNA-based methods in aquatic systems can have drawbacks, e.g. concerning water turbidity and population density. In highly turbid waters, e.g. after heavy rain fall, problems arise due to fast clogging of the glass fibre filters (Wittwer et al. 2018a). High amounts of suspended matter decrease the filtered water volume and increase the concentration of potentially inhibitory substances with a negative impact on subsequent eDNA analysis and the estimation of *A. astaci* spore concentration. The assessment of population density or biomass is a major task in eDNA-based aquatic biomonitoring, which is hampered by many factors. Detectable eDNA concentrations can strongly vary with sex ratio (e.g. Dunn et al. 2017), species-specific eDNA excretion (Thomsen et al. 2012a), behavioural preferences (Takahara et al. 2012) or season (Goldberg et al. 2011) and thus estimates for population density can easily be distorted. With higher water

turbidity and lower population density, the effort to recover enough genetic material (here: spores) for reliable eDNA detection increases. This includes the need for more samples per site to reach sufficient water volumes and subsequent pooling of eDNA extracts, which increases time and costs in the field and the laboratory.

While our eDNA approach leads to robust spore quantification in standing and running waters, even higher *A. astaci* detection probabilities in low population densities of infected crayfish may potentially be achieved by testing alternative detection devices such as digital PCR (Nathan et al. 2014) or new ways of water sampling (e.g. automated systems in combination with biosensors, Kearns et al. 2008). Moreover, the development of new marker systems, which combine the detection of invasive crayfish and *A. astaci* would strongly facilitate monitoring and management of these species (e.g. Robinson et al. 2018). A combined eDNA-based tool to discriminate between a wide range of invasive and native crayfish species as well as *A. astaci* could lead to a more complete understanding of invasion processes and migration pathways. This would allow to conclude on the type of infected crayfish species, the presence of indigenous crayfish and a possible threat of *A. astaci* transmission. First successful attempts to detect crayfish via eDNA have been achieved for invasive species such as *Pacifastcus leniusculus* (Agersnap et al. 2017; Dunn et al. 2017; Larson et al. 2017), *Orconectes rusticus* (Dougherty et al. 2016; Larson et al. 2017) and *Procambarus clarkii* (Cai et al. 2017; Tréguier et al. 2014; Mauvisseau et al. 2018) and for the endangered species *Astacus astacus* and *A. leptodactylus* (Agersnap et al. 2017), which should facilitate such a development.

In conclusion, eDNA-based *A. astaci* detection allowed us to estimate the range, density and distribution patterns of *A. astaci*-positive crayfish populations occurring in three different water courses. We found that estimated spore concentrations in eDNA samples correlate positively with population densities and pathogen loads of *A. astaci*-positive crayfish populations obtained from conventional trapping approaches. Both methods showed similar detection probabilities, with the eDNA assay being more sensitive in *A. astaci* detection.We also found that spores are quantifiable up to three kilometres downstream of a hot spot area of infected crayfish populations. The eDNA method as used in this study proved suitable on the detection of *A. astaci* infection sources (aquafarm) and migration fronts of infected crayfish close to refugial areas of native crayfish. Thus, we urge the use of this fast and reliable technology in a variety of applications regarding freshwater management and biomonitoring.

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Compliance with ethical standards

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

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

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Publication III – Supplementary Material

Supplement Table 1 Overview over study sites.

Study site	Subsites		Sample site coordinates	Distance and total length	Sampling month	Sampling depth
Ulmbach	8	A	N50.61887 E8.19237	**Ø 2.6 km**	August 2013	surface
		B	N50.60990 E8.25585	~5.4 km		
		C	N50.59653 E8.28078	~3.4 km		
		D	N50.58123 E8.30114	~2.9 km		
		E	N50.57154 E8.29413	~1.3 km		
		F	N50.56154 E8.29210	~1.2 km		
		G	N50.55025 E8.29632	~1.6 km		
		H	N50.53248 E8.30900	~2.4 km		
				Total: 18.2 km		
Lütter	8	A	N50.48746 E9.89498	**Ø 1.7 km**	August 2013	surface
		B	N50.49438 E9.88392	~1.4 km		
		C	N50.48924 E9.87235	~1.2 km		
		D	N50.49102 E9.85442	~1.6 km		
		E	N50.49055 E9.83887	~1.3 km		
		F	N50.48424 E9.81823	~2.1 km		
		G	N50.48141 E9.80131	~1.5 km		
		H	N50.47994 E9.76901	~3.0 km		
				Total: 12.1 km		
Weschnitz	8	A	N49.63963 E8.75975	**Ø 2.0 km**	August 2013	surface
		B	N49.62818 E8.76242	~1.4 km		
		C	N49.64061 E8.76776	~1.6 km		
		D	N49.61138 E8.75218	(B-D)		
		E	N49.60482 E8.74276	~1.2 km		
		F	N49.58919 E8.73090	~2.5 km		
		G	N49.57849 E8.71923	~1.7 km		
		H	N49.56090 E8.69519	~3.1 km		
				Total: 13.8 km		

Study site	Subsites		Sample site coordinates	Distance and total length	Sampling month	Sampling depth
Aquafarm (Lauter/Wetter system)	8	A	N50.55649 E8.95929	**Ø 265 m**	August 2014	Stream: surface Ponds: 10 cm above bottom
				500 m		
		B	N50.55264 E8.95835			
				260 m		
		C (pond)	N50.55222 E8.95909	(B-E)		
		D (pond)	N50.55203 E8.96096			
		E	N50.55060 E8.95791			
				470 m		
		F	N50.54830 E8.95351			
				25 m		
		G	N50.54809 E8.95228			
				70 m		
		H	N50.54770 E 8.95325			
				Total: 1.3 km		
NCA (Mümling-Rehbach system)	6	A	N49.69995 E8.96719	**Ø 530 m**	September 2014	Stream: surface Ponds: 10 cm above bottom
				360 m		
		B	N49.70138 E8.96278			
				1000 m		
		C (pond)	N49.70095 E8.95893	(B-D)		
		D (pond)	N49.70608 E8.95216			
				660 m		
		E	N49.70325 E8.94419			
				100 m		
		F (pond)	N49.70314 E8.94344			
				Total: 2.1 km		

Supplement Table 2 Sex ratio and mean carapax length of captured crayfish.

Location	Sex ratio [%]		Mean carapax length [cm ± SD]	
	♂	♀	♂	♀
Ulmbach	26	74	4.35 ± 0.77	3.96 ± 0.78
Lütter	41	59	4.05 ± 1.43	4.24 ± 0.79
Weschnitz	42	58	4.71 ± 0.95	4.85 ± 0.84
Aquafarm	44	56	4 ± 1.06	4.16 ± 0.71

Supplement Table 3: Filtered water volumes (in L). Volumes marked with * were achieved with two and ** with four pooled subsamples.

Site	Sampling area				
	Ulmbach	**Lütter**	**Weschnitz**	**Aquafarm**	**NCA**
A	5	10	3.5	3.5	3.5
B	4	10	3.5	3.5	2.5**
C	8.5	10	10	3.5*	3.5**
D	10	10	5	3*	1.6**
E	10	10	3.5	3.5*	7
F	10	10	3.5	3.5*	2.5**
G	10	10	7	6*	/
H	7.5	10	3.5	3.5	/

Summary (in German)

Hintergrund

Weltweit ist die Biodiversität aufgrund von natürlichen und anthropogenen Stressoren bedroht. Die Hauptgründe dafür liegen in der Zerstörung und Fragmentierung von Lebensräumen, der Überbeanspruchung natürlicher Ressourcen, der Umweltverschmutzung, der Einführung gebietsfremder, invasiver Arten sowie in sich wandelnden klimatischen Verhältnissen. Angesichts der großen Bedeutung der biologischen Vielfalt für das moderne menschliche Leben sind effiziente Schutzstrategien zum Erhalt der natürlichen Lebensräume und Arten dringend erforderlich. Zu diesem Zweck wurden ehrgeizige multilaterale Abkommen auf regionaler und globaler Ebene beschlossen, die den weiteren Verlust an biologischer Vielfalt verhindern sollen.

Ein effizientes Biomonitoring ist erforderlich, um die Kriterien der gesetzlich verbindlichen Konventionen zu erfüllen. Das Artenmonitoring als Kernaktivität der Biodiversitätsforschung ist ein wirksames Instrument, um den aktuellen Status einer Art sowie Populationstrends in einem bestimmten Lebensraum zu bewerten. Die gesammelten Daten werden anschließend für die Entwicklung von Schutz- und Erhaltungsmaßnahmen verwendet. Dies kann durch visuelle, elektronische oder genetische Überwachungsmethoden erreicht werden. Derzeitige Mängel beim Biomonitoring beziehen sich auf eine unzureichende Erfassung der Arten. Mittels neuer, auf „Umwelt-DNA" (*environmental DNA* oder eDNA)- basierender Verfahren lassen sich bereits kleinste DNA-Spuren in Umweltproben (Boden-, Sediment-, Wasser- und Luftprobe) nachweisen. Diese Technologie eröffnet neue Möglichkeiten der Artenerfassung und des Biodiversitätsschutzes. Die eDNA-Methodik ermöglicht die qualitative (Präsenz/Absenz) und (semi-) quantitative Detektion von Einzelarten. Auch die Bestimmung der Zusammensetzung von Artgemeinschaften ist über eDNA-Metabarcoding-Ansätze möglich.

In dieser Arbeit wird ein eDNA-basierter Nachweis von *Aphanomyces astaci* (Schikora 1906) angewandt. Der Oomycet *A. astaci* verursacht die Krebspest, eine todbringende Krankheit unter europäischen Flusskrebsarten. Gegen Ende des 19. Jahrhunderts wurde *A. astaci* durch nordamerikanische Flusskrebsarten wie beispielsweise dem Signalkrebs *Pacifastacus leniusculus* nach Europa eingeschleppt. Als latente Träger sind sie zumindest teilresistent gegenüber einer *A. astaci*- Infektion. Sie übertragen den

hochinfektiösen Krebspesterreger an die einheimischen Flusskrebsarten, die innerhalb kürzester Zeit sterben. In Europa sind besonders der Edelkrebs *Astacus astacus* (Linnaeus 1758), der Steinkrebs *Austropotamobius torrentium* (Paula Schrank 1803) sowie der Dohlenkrebs *Austropotamobius pallipes* (Lereboullet 1858) gefährdet. Die weitere Ausbreitung gebietsfremder, invasiver Krebsarten sowie *A. astaci* führt zu einer schnellen und weitgehenden Zurückdrängung einheimischer Flusskrebsarten in europäischen Gewässern. Die Überwachung und Eindämmung dieses Pathogens ist für den Schutz einheimischer Flusskrebsarten von höchster Wichtigkeit.

Fragestellung und Ziele

Die von Strand et al. (2011, 2012, 2014) entwickelte eDNA-basierte Methode zur Detektion von *A. astaci* ermöglicht einen nichtinvasiven, direkten Nachweis des Erregers über dessen Sporenfreisetzung in das umgebende Wasser. In dieser Arbeit wird die Methodik von Strand et al. (2014) angewandt, um weitere Einblicke in die Ökologie, Überlebensfähigkeit und Sporulationsdynamik von *A. astaci* unter natürlichen Bedingungen zu erhalten. Diese Arbeit an der Schnittstelle zwischen experimenteller und angewandter Forschung evaluiert zudem, ob sich die Methodik für eine großangelegte *A. astaci*-Überwachung sowie zum Risikomanagement gefährdeter Gewässerabschnitte eignet. Dafür sollen in dieser Arbeit folgende Fragen beantwortet werden:

1. Ermöglicht die eDNA-basierte Methodik die Ermittlung einer räumlichen und saisonalen Variation der *A. astaci* Sporulationsdynamik in natürlichen Gewässersystemen (**Publikationen I, II, III**)? Ist sie außerdem in der Lage, den Transport von *A. astaci*- Sporen in Fließgewässern abzubilden (**Publikation III**)?
2. Welche der zwei bekannten eDNA-Verfahren zur Detektion von *A. astaci* ist das vielversprechendste Wasserfiltrationsverfahren (**Publikation II**)?
3. Ermöglicht das eDNA-basierte Nachweisverfahren die Ermittlung von *A. astaci*-Sporenkonzentrationen bei unterschiedlicher Populationsdichte und/oder Pathogenbelastung infizierter Krebse (**Publikation III**)?
4. Können eDNA-Nachweise als alleinige Nachweismethoden genutzt werden oder stellen sie eher sinnvolle Ergänzungen für konventionelle Biomonitoring-Verfahren dar (**Publikation I** und **III**)?

Durch Beantwortung dieser Fragen soll ein wichtiger Beitrag zur praktischen Verwendung von eDNA-Methoden im angewandten Naturschutz geleistet werden.

Zusammenfassende Ergebnisse

Publikation I

Zur Untersuchung des Einflusses saisonal schwankender Wassertemperaturen auf die Sporulationsdynamik wurde ein ganzjähriges *A. astaci* eDNA-Monitoring sowie parallele Flusskrebs-Bereusungen in einem Gewässersystem mit *A. astaci*-positiven Signalkrebspopulation durchgeführt. Dabei konnte gezeigt werden, dass die Sporulationsdynamik von *A. astaci* durch saisonale variierende Wassertemperaturen und lebenszyklusbedingte Faktoren infizierter Krebse (Häutung, Aktivität, Paarung) beeinflusst wird. Da *A. astaci*-DNA über das gesamte Jahr, auch im Winter, mittels eDNA nachweisbar war, ist davon auszugehen, dass zu jeder Jahreszeit ein hohes Übertragungsrisiko von *A. astaci*-Sporen auf andere Gewässer gegeben ist. Diese Studie zeigte zudem, dass der eDNA-basierte Nachweis von *A. astaci* eine gute Ergänzung für konventionelles Biomonitoring mittels Fangtechniken darstellt z.B. aufgrund längerer Detektionszeiten und einem geringerem Zeitaufwand in Feld und Labor.

Publikation II

Durch die Erfassung von Vergleichsdaten zweier verschiedener eDNA-Methoden (Dead-End-Ultrafiltration, DEUF; Tiefenfiltration, DF) sollte das vielversprechendste Wasserfiltrationsverfahren für den Nachweis von *A. astaci* evaluiert werden. Diese Evaluation erfolgte anhand von drei unterschiedlichen Gewässersystemen sowie zwei Jahreszeiten (Frühling, Sommer). Obwohl beide eDNA-Methoden *A. astaci*-Sporen erfolgreich nachweisen konnten, zeigten die Vergleichsdaten unterschiedliche Nachweiswahrscheinlichkeiten in Abhängigkeit von der Jahreszeit, die hauptsächlich von saisonbedingter Wassertrübung beeinflusst wurden. Es zeigten sich zudem methodenspezifische Vor- und Nachteile in der Anwendbarkeit. Als Resultat eignet sich die etwas sensitivere, aber deutlich anspruchsvollere DEUF-Methodik für die Untersuchungen von speziellen Probestandorten (z.B. nahe von Krebssperren). Die DF-Methode eignet sich aufgrund der schnellen, kostengünstigen und unkomplizierten Handhabung für großangelegte eDNA Biomonitoringmaßnahmen.

Publikation III

Der eDNA-basierte *A. astaci*-Nachweis soll zukünftig für großangelegte Biomonitoringmaßnahmen und Risikomanagement genutzt werden. Dafür müssen mögliche Auswirkungen unterschiedlicher Populationsdichten und/oder Pathogenbelastungen infizierter Krebspopulationen auf dessen Detektierbarkeit in Wasserproben getestet werden. Dies erfolgte mit paralleler eDNA-Probenahme und Flusskrebs-Bereusung in Gewässersystemen mit invasiven Krebspopulationen in verschiedenen Invasionsstadien (etablierte Populationen, kürzlich eingeführte Individuen, schnelle Verbreitung flussaufwärts). Außerdem wurde der parallele Beprobungsansatz nahe Bereichen mit erhöhtem Übertragungsrisiko (signalkrebszüchtende Fischfarm) und innerhalb von Schutzgebieten für einheimische Flusskrebsarten durchgeführt. In allen getesteten Fällen ermöglichte die eDNA-Methodik die Erfassung des Vorkommens von *A. astaci*, selbst bei sehr geringen Populationsdichten und Pathogenbelastungen. Die Ergebnisse der eDNA-basierten *A.* astaci-Detektion waren vergleichbar mit den durch konventionelle Fangtechnik erhaltenen Resultaten. Die Ergebnisse zeigte darüber hinaus die räumlich variierende Detektierbarkeit von *A. astaci* in Wasserproben. Die geschätzte Sporenkonzentration korrelierte dabei positiv mit der Populationsdichte von infizierten Flusskrebspopulationen, wodurch eine Einschätzung bezüglich ihrer Ausbreitungsschwerpunkte und Ausbreitungsgrenzen in natürlichen Gewässersystemen ermöglicht wird. Desweiteren konnte gezeigt werden, dass die individuell variierende Pathogenbelastung einzelner Krebse den Sporengehalt in eDNA Proben unter natürlichen Bedingungen beeinflusst. Außerdem konnte ein Transport von *A. astaci*-Sporen flussabwärts von ca. 3 km mittels eDNA nachgewiesen werden.

Fazit zum eDNA-basierten A. astaci-Nachweis

Zum Schutz der europäischen Flusskrebse werden zuverlässigen Informationen über das Vorkommen von *A. astaci* in natürlichen Gewässern benötigt. Zudem ist das Wissen über zeitliche, räumliche und individuelle Variationen der Sporulationsdynamik hilfreich, um effiziente Managementmaßnahmen einzuleiten.

Die Resultate dieser Arbeit zeigen, dass das eDNA-basierte Monitoring bereits heute den Nachweis von *A. astaci* in chronisch infizierten Krebspopulationen, während oder nach akuten Krebspest-Ausbrüchen sowie in frühen Invasionsstadien invasiver, *A. astaci*-positiver Flusskrebsarten ermöglicht. In Zukunft könnte eine umfassende eDNA-

Überwachung natürlicher Wassersysteme zur Frühwarnung installiert werden. Auch bei der Suche nach geeigneten Wiederbesiedlungsgebieten einheimischer Flusskrebse kann die eDNA-Methodik hilfreich sein. Dabei können geeignete Lebensräume als „*A. astaci*-frei“ verifiziert oder angrenzende Gewässersysteme nach infizierten invasiven Krebsarten abgesucht werden.

eDNA-basiertes Monitoring kann zur Kontrolle der weiteren Ausbreitung von *A. astaci* verwendet werden, u.a. zur Überwachung der Abnahme von Zoosporen-Konzentrationen im Wasser nach akuten Ausbrüchen oder nach Entnahme infizierter Krebse aus Gewässern. Zudem ermöglicht das eDNA Monitoring die Untersuchung des Zoosporen-Transports in und zwischen Gewässern. Dadurch könnten neue Ausbrüche vorhergesagt oder mit entsprechenden Gegenmaßnahmen verhindert werden. Auch die Überwachung des Einlass- und Auslasswassers in Fischzuchten, die gebietsfremde Flusskrebsarten kultivieren, wird durch diese Methodik ermöglicht.

Generelles Fazit

Der weltweit rapide Verlust an biologischer Vielfalt aufgrund verschiedener natürlicher und anthropogener Ursachen erfordert effiziente Schutz-und Erhaltungsmaßnahmen. Theoretisch und experimentell hat sich die eDNA-Methodik als geeignetes Monitoringinstrument erwiesen, allerdings scheint es Probleme bei der praktischen Umsetzung im angewandten Biomonitoring zu geben. Diese können in der nachwievor erfolgenden Grundlagenforschung zur Ökologie der eDNA oder einzelnen Arbeitsschritten von Probenahme bis Analyse liegen. Um als Standardüberwachungsmethode genutzt werden zu können, müssen zudem Probleme wie bespielsweise falsch-positive und falsch-negative Detektionen ausgeräumt werden.

Trotz der derzeitigen Einschränkungen eignen sich eDNA-basierte Methoden, ob als Einzelartennachweis oder als Metabarcoding-Verfahren, als sinnvolle Ergänzung für konventionelle Monitoringmethoden. Kombinierte Ansätze von eDNA- und konventionellen Methoden werden zu einem umfassenderen Verständnis der Biodiversität und der Funktionsweise von Ökosystemen führen. Langfristig ist damit zu rechnen, dass die eDNA-Methodik einen wichtigen Beitrag zum Schutz der Biodiversität leisten und die praktische Umsetzung von Biodiversitäts-Schutzabkommen unterstützen wird.

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