Detection of crayfish plague spores in large freshwater systems

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Summary

1. Indigenous European freshwater crayfish (ICS) are threatened due to invasive North American freshwater crayfish that are natural carriers of *Aphanomyces astaci* which causes crayfish plague. Infectious *A. astaci* zoospores are released from carrier crayfish, but little is known about the spore abundance in water systems that either host non-indigenous crayfish species (NICS) or experience crayfish plague outbreaks. We tested two large-scale filtering approaches to generate new insight about the abundance and dynamics of *A. astaci* spores in natural freshwater systems.

2. Depth filtration (DF) and dead-end ultrafiltration (DEUF) followed by *A. astaci*-specific quantitative real-time PCR was used to monitor *A. astaci* spores in large Nordic lakes hosting *A. astaci*-positive *Pacifastacus leniusculus*, the dominating NICS in Northern Europe. Crayfish and water were sampled together to compare the *A. astaci* pathogen load in tissues, *A. astaci* prevalence in the population and the corresponding spore density in water. Samples were also obtained from a river where indigenous noble crayfish suffered from acute crayfish plague. The sensitivity of the filtering techniques was evaluated using simulation of random events.

3. We detected *A. astaci* spores in lakes hosting NICS with both filtering methods but predominantly at concentrations below *c.* 1 spore L^{-1}. We found a significant positive association between *A. astaci* spore density in water, the *A. astaci* prevalence in the corresponding NICS population and the tissue pathogen load. Water from the river with the ongoing crayfish plague outbreak contained overall *c.* 43 times more spores L^{-1} than water hosting NICS. Both filtering techniques proved suitable and equally sensitive, but simulations suggest that an optimization of the spore recovery could yield a 10-fold increase in the DEUF-method sensitivity.

4. *Synthesis and application.* Our study demonstrates a low amount of pathogen spores are present in aquatic environments with non-indigenous crayfish species, emphasizing the need for large-volume filtering techniques for successful detection. The approach can be used for risk assessments and to improve conservation and management strategies of crayfish in Europe. Applications of this method include targeted disease surveillance, habitat evaluation prior to crayfish re-stockings and water monitoring that can minimize disease transmission and spread, for example in crayfish farms and prior to fish movements for stocking purposes.

Key-words: alien species, *Aphanomyces astaci*, dead-end ultrafiltration, depth filtration, disease surveillance, environmental DNA, environmental monitoring, glass fibre filter, *Pacifastacus leniusculus*, signal crayfish

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Introduction

Introduction of non-indigenous crayfish species (NICS) is the largest threat to indigenous crayfish species (ICS), either as a result of competitive exclusion or disease agents that are transmitted and spread by the invaders (Holdich *et al.* 2009). The specialized freshwater crayfish parasite *Aphanomyces astaci* Schikora (Saprolegniales, Oomycota) lives in a balanced host–parasite relationship with North American crayfish, but acts as a lethal pathogen causing the crayfish plague in European ICS (Söderhäll & Cerenius 1999). Of the nine NICS established in Europe, the three most common (signal crayfish *Pacifastacus leniusculus* Dana, 1852; spiny cheek crayfish *Orconectes limosus* Rafinesque, 1817; and red swamp crayfish *Procambarus clarkii* Girard, 1852) are confirmed carriers of *A. astaci* (Diéguez-Uribeondo 2006). Recently, *A. astaci* has been detected in another NICS in Europe the calico crayfish *Orconectes immunis* Hagen, 1870 (Filipová *et al.* 2013; Schrimpf *et al.* 2013a). Unless intensive proactive conservation measures are taken to protect the European species, these NICS may dominate in the next few decades (Holdich *et al.* 2009). Improved tools for disease monitoring and control are therefore urgently needed to aid crayfish conservation and management.

The transmission of *A. astaci* occurs through motile zoospores that locate crayfish hosts using chemotaxis and thereafter penetrate the crayfish cuticle (Söderhäll & Cerenius 1999). Little is known about the abundance and dynamics of *A. astaci* spores in water under natural conditions, except that a drastic increase in spore numbers is expected under crayfish plague outbreaks in ICS populations (Makkonen *et al.* 2013). Two recent studies (Strand *et al.* 2012; Svoboda *et al.* 2013) revealed that *A. astaci* spores are frequently released from infected carrier NICS, with increased spore release in concurrence with moulting events and death. Even at low temperatures (4 °C), *A. astaci* spores were frequently released from the carriers (Strand *et al.* 2012). Therefore, a continuous and highly variable presence of infective *A. astaci* spores can be expected in waters hosting *A. astaci*-positive NICS.

Quantitative real-time PCR (qPCR) is widely used to detect and determine the levels of viruses (He & Jiang 2005), bacteria (Santo Domingo, Siefring & Haugland 2003), protozoan pathogens (Guy *et al.* 2003; Varma *et al.* 2003) and other micro-organism in water matrices and environmental samples. Further, qPCR and other DNA-based approaches can also be used to monitor non-indigenous or endangered macro-organisms (Darling & Mahon 2011; Lodge *et al.* 2012; Thomsen *et al.* 2012). Strand *et al.* (2011) obtained a minimum detection level of one *A. astaci* spore from spiked environmental water samples using membrane filters and successfully detected low amounts of *A. astaci* spores in small ponds containing signal crayfish. However, recent results showed that a series of ≤1 L water samples from a Norwegian lake hosting *A. astaci*-positive signal crayfish failed to capture *A. astaci* spores in almost all of the *c.* 65 samples (Strand 2013). Thus, small water volumes appear to be insufficient for monitoring of *A. astaci* spores in large water systems with NICS. Depth filters (DF) typically exhibit a high particle-holding capacity compared to membrane filters and can therefore process larger water volumes. Ultrafiltration using hollow-fibre filters may be even more effective to process large-volume water samples for concentration and recovery of different microbes (Lindquist *et al.* 2007; Smith & Hill 2009).

The purposes of our study were to (i) explore two large-scale filtration methods for operational monitoring of *A. astaci* under field conditions, (ii) reveal temporal and spatial sporulation patterns of *A. astaci* in natural habitats, (iii) compare *A. astaci* prevalence in signal crayfish populations and the ambient water, and if the opportunity arose (iv) explore the *A. astaci* sporulation intensity during a crayfish plague outbreak. Here, we report the results from field surveys in Finland, Sweden and Norway, where we found a significant positive association between *A. astaci* spore density in the water and *A. astaci* prevalence in the carrier population. Further, the sporulation intensity during and shortly after a crayfish plague outbreak was prominent compared to the very low spore levels that were found in waters hosting NICS, that is, often below 1 spore L^{-1}. The latter result demonstrates that a lethal alien parasite can go undetected with monitoring approaches relying on small water volumes. Hence, the ability to specifically monitor and quantify infrequent crayfish plague spores in natural water sources may pave the way for improved conservation and management strategies of freshwater crayfish in Europe.

Materials and methods

STUDY SITES

Five sites (A–E) were included in this study (Table 1) and consisted of (A) two outdoor ponds (A1–2) at a signal crayfish farm in Orivesi (Finland), (B) two subsites (B1–2) of Lake Saimaa (Finland), that hosts a signal crayfish population of known *A. astaci* carrier status (Strand *et al.* 2011), (C) two subsites (C1–2) of the small river Mäntyjoki (Finland) where an acute crayfish plague outbreak was identified in early August 2010 in a noble crayfish population, (D) three subsites (D1–3) of Lake Stora Le (Sweden) and (E), three subsites (E1–3) of the neighbouring Lake Øymarksjøen (Norway) that both host signal crayfish with known positive *A. astaci* carrier status (Vrålstad *et al.* 2011).

WATER SAMPLING AND FILTRATION TECHNIQUES

Water samples from the three first study sites (the Finnish farm, lake and river) were collected using depth filtration (DF, see Fig. S1, Supporting Information), while water from the Swedish and Norwegian lakes were collected using dead-end ultrafiltration (DEUF, Fig. S1, Supporting Information). For the Finnish farm and lake, 10 water samples were collected per site (five samples per subsite) at each sampling date. Water from *c.* 10 cm above the bottom was pumped into separate water containers (15 L),

Table 1. Overview over study sites

Study site	Subsite	Size	GPS*	Sampling period	Sample events	Sampling depth	Total sample volume
A: Farm Orivesi, Finland	A1	1300 m³	61·59038 24·23042	June–September 2010	8	10 cm ab	25 L × 8
	A2	1500 m³	61·59038 24·23042				
B: Lake Saimaa, Finland	B1		61·16207, 28·50214	June–September 2010	6	10 cm ab (1 ½ m)	75 L × 6
	B2		61·16287, 28·50165			10 cm ab (6 m)	
C: River Mäntyjoki, Finland	C1		63·06286, 28·71973	August–September 2010	4	Surface	25 L × 4
	C2		63·06443, 28·71634	September 2010	2		25 L × 2
D: Lake Stora Le, Sweden	D1		59·15590, 11·87142	May–October 2011	6	1 m bs, 20 cm ab	200 L × 6
	D2		59·18197, 11·83833				
	D3		59·18817, 11·82675				
E: Lake Øymarksjøen, Norway	E1		59·33433, 11·64094	May–October 2011	6	1 m bs, 20 cm ab	200 L × 6
	E2		59·32964, 11·64478				
	E3		59·32375, 11·66056				

bs, below surface; and ab, above bottom.
*Degree decimal.

using a battery driven bilge pump (TMC-03304, 12V) connected to a hose (18 mm inner diameter). The whole or part of the water sample was transferred into a Millipore stainless steel pressure container (5 and 15 L samples from the farm and lake, respectively) and were filtered on-site into a glass fibre filter (AP25, 47 mm diameter; Millipore, Billerica, Massachusetts, USA) using a 47-mm in-line filter holder (Millipore), with air pressure of max 5 bar regulated (AGA Unicontrol 500, AGA, Oslo, Norway) from a 5-L 200-bar air tank. The filtered water volume was measured for each sample and ranged between 2–15 L depending on the turbidity. Overall, *c.* 4·7 and 8·3 L was filtered per sample from the farm and lake, respectively. Each glass fibre filter was transferred to a 15-mL Falcon tube and kept on ice until transfer to the laboratory where stored at −20 °C.

For the Finnish river, the crayfish plague was identified (isolation and qPCR) at subsite C1 and moved upstream (C2) before it reached the noble crayfish-inhabited lake Mäntyjärvi in late September. Water samples were collected from C1 four times during August and September and C2 twice in September. At the first sampling event at both sites, a few dead or moribund noble crayfish were seen on the bottom of the shallow stream. Five water samples were collected at each subsite and sample event using 5-L containers that were submerged directly into the stream. The containers were stored overnight at 4 °C, and the water was filtered as described above. In several cases, a water sample was filtered onto two glass fibre filters due to filter clogging; overall *c.* 3·1 L (between 1·5 and 5 L) was filtered per filter. Each filter was transferred to a 15-mL Falcon tube and stored at −20 °C.

For each subsite in the Swedish and Norwegian lakes (D1–3 and E1–3, respectively), water was pumped in parallel into separate Rexeed™-18AX (Asahi Kasei, Tokyo, Japan) hemodialyse filters (1·8 m² surface area, *c.* 30 kDa pore size) following the dead-end filtration set-up described in Smith & Hill (2009) using a peristaltic pump (Cole-Parmer Masterflex L/S pump, Cole-Parmer, Vernon Hills, Illinois, USA) powered with a 1000 W aggregate. The internal pressure was kept at *c.* 172 368 Pa, and 100 L was filtered over 60–90 min. The filtering process was carried out from a boat, and parallel samples were taken from approximately 20 cm above the bottom (1·5–5 m depth) and 1 m below the water surface. For each parallel sample, the inlet hose was attached to a plastic box (36 × 29 × 21 cm) covered with a plankton net (32·5 × 26 cm, 90 μm mesh) and weighted with 2 kg of lead. This prevented large particles from entering and

clogging the ultrafilters that are composed of hollow fibres, each with a diameter of 185 μm. To broaden the sample area at each subsite, 50 L of water was passed through each filter before the hose inlet was moved horizontally 10 m to filter another 50 L of water. Sodium azide was added to each filter to a final concentration of 0·05% to terminate ongoing biological activity within the filters. The filter samples were stored and transported in an insulated box with cooling units and processed in the laboratory within 36 h of sampling.

The ultrafilter contents were eluded using 300 mL of 0·001% TWEEN® 80 (Merck Schuchardt OHG, Hohenbrunn, Germany) solution while forward flushing the filters into sterile glass flasks using the peristaltic pump. The residual volume of 100 mL was then collected using backward flushing, resulting in *c.* 400 mL suspension from each filter. The samples were divided into two 250-mL polypropylene bottles and centrifuged for 10 min at 9600 × *g* to pellet all particles including putative *A. astaci* spores. The supernatant was discarded, and the pellet transferred to a 50-mL Falcon tube and frozen at −80 °C following freeze drying. In retrospect, in a few cases, we also retained and analysed the supernatant separately by qPCR to test whether some *A. astaci* spores retained in the supernatant. The weight (Mean ± SD) of the freeze-dried pellets (dry weight of the recovered seston) was higher for the Norwegian lake (221·7 ± 37·4 mg) than for the Swedish lake (30·1 ± 7·1 mg).

CRAYFISH SAMPLES

Permission for collecting signal crayfish and environmental samples from *A. astaci* contaminated waters was obtained where needed, and all equipment was disinfected after each sampling event. Catch per unit effort (CPUE, crayfish per trap per night) was estimated for the three lakes using ten baited traps per subsite per sampling event. Ten signal crayfish were collected from the baited traps from the farm and lakes at each sample event, but not from the river. The crayfish were transported on ice and then frozen in −20 °C (Finnish sites) or euthanized in 96% ethanol on-site (Norwegian and Swedish sites). From each crayfish, the telson and two uropods were collected as one sample. Visible melanized spots were also dissected from the crayfish when present. The acute crayfish plague was diagnosed in the noble crayfish from the river Mäntyjoki by isolation of *A. astaci* in pure culture (following Viljamaa-Dirks & Heinikainen 2006) followed by PCR

confirmation (following Oidtmann *et al.* 2006) at C1 and by qPCR (following Vrålstad *et al.* 2009) at C2.

DNA EXTRACTION

DNA extraction of crayfish tissues followed Vrålstad *et al.* (2009). For DF samples, we added 4 mL preheated (65 °C) CTAB buffer (20 g L^{-1} CTAB, 1·4 M NaCl, 0·1 M Tris–HCl, 20 mM Na_2 EDTA) with 1% 2-Mercaptoethanol to the Falcon tube containing the glass fibre filter and ruptured the filters in the buffer using long pipette tips. To further facilitate cell fracturing, the sample was frozen (−80 °C, 30 min), thawed (65 °C), 40 μL proteinase K (20 mg mL^{-1}) was added, and the sample was then incubated at 65 °C for 1 h. Chloroform (3 mL) was added, and the tubes were shaken manually for *c.* 15 s before centrifugation (15 min, 3800 × *g*). For each sample, 2 × 1200 μL of the supernatant was transferred to Eppendorf tubes (2 mL), yielding two subsamples per filter. The samples were incubated at 37 °C (30 min) with 10 μL RNase (10 mg mL^{-1}) followed by RNase deactivation on ice for 5 min. Ice-cold isopropanol (720 μL) was added, followed by tube inversion and incubating (room temperature, 15 min). The tubes were centrifuged (16 000 × *g*, 15 min), and the supernatant was carefully removed. The pellet was then washed with 300 μL of 70% ethanol and centrifuged (16 000 × *g*) for 5 min. The ethanol was carefully removed and the DNA pellet dried (Heto Holten DNA Mini Centrifugal Evaporator, Heto Holten, Allerød, Denmark) before re-suspension in 100 μL TE buffer.

The whole or parts of the freeze-dried pellets from DEUF samples (≤100 mg) were used for DNA extraction. The sample was transferred to disposable 2-mL tubes with steel beads (Kit Precellys MK28, Bertin Technologies, Montigny-le-Bretonneux, France), 1 mL preheated CTAB buffer with 1% 2-Mercaptoethanol was added, and samples were homogenized on a Precellys 24 lysis and homogenization automated equipment (Bertin technologies, Montigny, France) with the program 6500 rpm (1 min) × 2 sessions. The subsequent DNA isolation protocol followed the description above, apart from using 10 μL proteinase K (20 mg mL^{-1}), 600 μL chloroform and 1 × 1000 μL supernatant.

QUANTITATIVE REAL-TIME PCR

An *A. astaci*-specific qPCR assay was used according to Vrålstad *et al.* (2009) to detect and quantify *A. astaci* in crayfish and water samples on a Mx3005P qPCR system (Stratagene) with a modified temperature cycle (62 °C annealing, 30 s) to increase the assay specificity (Strand 2013). The DF samples were run in quadruple with two undiluted and two 10-fold diluted templates, while the DEUF samples were run in quadruple with two 10-fold and 100-fold diluted templates due to severe PCR inhibition if using the undiluted template DNA. The crayfish samples were run in duplicate: undiluted and 10-fold diluted. Environmental control and extraction control (for each DNA extraction run) were included in all qPCR tests. The mean PCR forming unit (PFU; Vrålstad *et al.* 2009) value per sample was estimated from both of the undiluted and diluted DNA sample, or just the diluted sample in case of inhibition (see Kozubíková *et al.* 2011). A sample result was only regarded as *A. astaci* positive if the overall detection (mean for all PCR-replicates) was above the limit of detection (LOD = 5 PFU, corresponding to ≥ agent level A2; Vrålstad *et al.* 2009).

The spore concentrations (spores L^{-1}) were estimated by multiplying the mean PFU value from the qPCR by 20 (1/20 part of

the DNA extract was used for the qPCR), and divided by 138 (the estimated median PFU value per spore; Strand *et al.* 2011), yielding a rough estimate of the spore content per sample. Finally, this number was divided with the amount of water filtered for that sample to obtain estimates of spores L^{-1}. These estimates assume a 100% recovery from the ultrafilters, but many aspects suggest the estimates from the DEUF samples probably underestimate the true spore concentration at the sampling point (see below).

STATISTICS

All statistical tests and model simulation were run in the software R v 2.15.1 (R Development Core Team, 2011). We used a negative binomial regression model (nb-glm) to analyse the *A. astaci* spore estimates (spores L^{-1}) from each location separately. The response variable was the spore estimate rounded to the number of whole spores per litre. For sites A and B, the following predictor variables and their possible two-way interactions were included in the model selection: surface temperature, weekly PFU per crayfish, weekly *A. astaci* prevalence in crayfish, week and subsite. For site D and E, the following variables were included in the model selection: surface temperature, weekly PFU per crayfish, weekly *A. astaci* prevalence in crayfish, CPUE, sampling depth, week, subsites and possible two-way interactions between these variables. The Akaike's information criterion (AIC) was used for model selection. Further, we tested for an association between the spore concentrations and the observed prevalence of *A. astaci*-positive signal crayfish population in the respective lakes using nb-glm. Finally, the spore concentrations detected during the crayfish plague outbreak were compared (using nb-glm) with the spore concentrations in the signal crayfish sites (both farm and lakes).

The probability to detect *A. astaci* spores at different concentrations in water was simulated using random events, assuming one, two or five repeated samples for the filtration set-ups. To account for a heterogeneous spore distribution, number of spores per litre was assumed to follow a negative binomial distribution with mean equal to assumed spore concentration, μ, and variance = $\mu + \mu^2$. The probability of spore detection was calculated as the proportion of 10 000 simulation runs for which there was calculated a positive qPCR signal. For the DF, the following parameters were assumed: 5 L sample volume; 5% loss of spores through the filter (corresponding to the manufacturers claim of 95% retention of particles; 0·9–8 μm); 10% loss during DNA extraction; and that at least three spores required in the initial sample to get positive qPCR detection as samples were divided into tree subsamples during DNA extraction. The recovery of particles from ultrafilters can be as low as 50% (Smith & Hill 2009; Mull & Hill 2012). We also experienced strong PCR inhibition in undiluted DNA templates from DEUF samples as well as suboptimal centrifugation procedure leading to loss of *A. astaci* spores that remained in the discarded supernatant (see Results, sensitivity of the filtering techniques). For the DEUF, we therefore assumed the following parameters: 100 L sample volume; the loss of spores during the recovery of the sample from the ultrafilter modelled by a beta-PERT distribution (a version of the beta distribution being specified by the three parameters: a minimum, maximum and most likely) with 30% loss as the most likely and range 5–50% (Smith & Hill 2009; Mull & Hill 2012); 50% loss during the centrifugation step; 10% loss during DNA extraction;

and at least 10 spores required to get a positive qPCR detection (PCR inhibition). Finally, we changed two of the parameters for the DEUF to evaluate how a putative optimized protocol would increase the likelihood of detecting spores: only 10% loss during the centrifugation step and only three spores required for positive qPCR detection.

Results

SIGNAL CRAYFISH FARM AT ORIVESI, FINLAND (SITE A)

The observed prevalence of *A. astaci*-positive signal crayfish ranged from 30% to 100% during the season (June–September) but was overall 78% (see Table S1, Supporting Information). The PFU level in tissue samples (median with 25th and 75th percentile) was 107 (5, 1625) corresponding to agent level A3 (A2, A4). A total of 56% of the 78 water samples were *A. astaci* positive. The selected predictor variables for the nb-glm describing spores L^{-1} were pond and temperature > 22 °C (binary variable). The threshold at 22 °C was suggested by explorative analyses by fitting a nonlinear spline function to temperature. There were significantly ($P < 0.001$, nb-glm) more spores in subsite A2, containing more crayfish than subsite A1 (Fig. 1a). Further, there were less spores at sample dates where the temperature rose above 22 °C in subsite A2 ($P < 0.001$, nb-glm). We detected up to *c.* 50 spores L^{-1} in subsite A2 during weeks 23–26, while relatively few spores were detected from weeks 28 onward (Fig. 1a).

LAKE SAIMAA, FINLAND (SITE B)

The overall observed prevalence of *A. astaci*-positive signal crayfish was 87% and varied from 80% to 100% during the season (weeks 27–35, Table S1, Supporting Information). The median PFU level in tissue samples was 978 (quartiles 166, 6010) corresponding to agent level A3 (A3, A4). A total of 92% of the 60 water samples analysed were *A. astaci* positive. The selected predictor variables in the nb-glm describing the spores L^{-1} were subsite, week (as factor) and the subsite–week interaction. We observed no difference ($P = 0.90$, nb-glm) in the overall spore concentration at the two subsites that ranged from 0·2 to 11 spores L^{-1} (Fig. 1b), where the CPUE were *c.* 2·8 (subsite B1) and *c.* 1·8 (subsite B2). However, the spore concentrations at subsite B2 were significantly lower for week 33 and 35 compared to subsite B1 and earlier sampling at B2.

RIVER MÄNTYJOKI, FINLAND (SITE C)

All 38 water samples from the river affected by crayfish plague were *A. astaci* positive. We observed high spore concentrations during the outbreak (20–500 spores L^{-1}) with a steady decline over time (Fig. 1c). The plague was identified at subsite C1 1 week before the first sampling and moribund and dead noble crayfish were observed
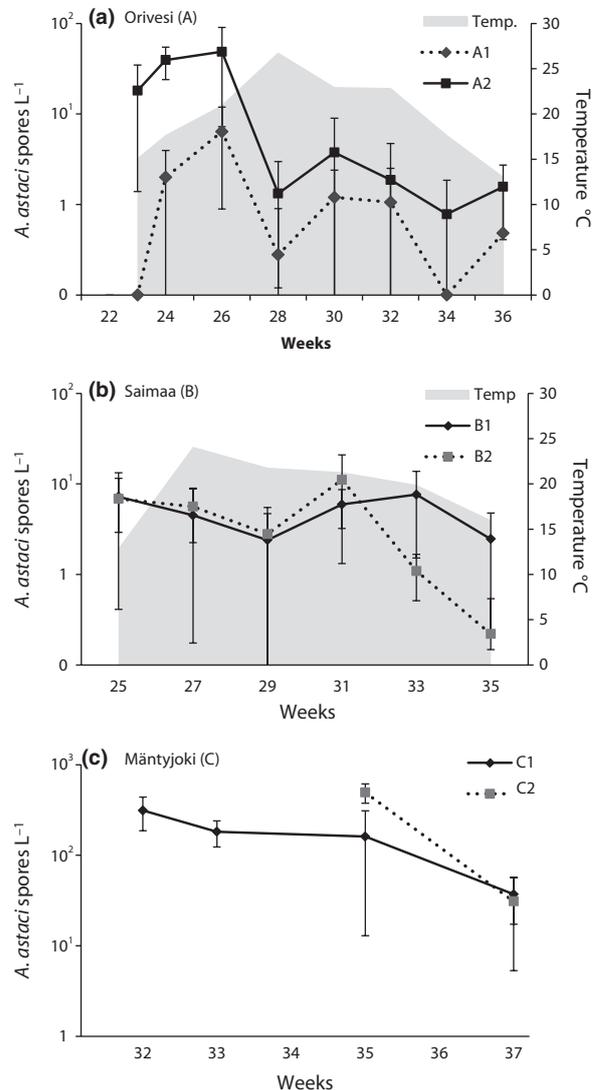

Fig. 1. Mean spore estimates (±SD) obtained from water samples collected from Finland: (a) The crayfish farm (A) and (b) Lake Saimaa (B) and (c) River Mäntyjoki (C) affected by the crayfish plague. Time of sampling is referred to as week numbers (June: 23–26, July: 27–30, August: 31–34, and September: 35–37).

during the first sampling day. The outbreak spread upstream, advancing in a similar manner as reported in Westman & Nylund (1978). When moribund noble crayfish were observed at subsite C2, water samples were collected there too. The highest amounts of spores were detected for both subsites during the first sampling event, which coincided with the front of the acute outbreak (Fig. 1c).

LAKE STORA LE, SWEDEN (SITE D)

The overall observed prevalence of *A. astaci*-positive signal crayfish was 49% (Table S1, Supporting Information) and varied from 20% to 80% during the season. In accordance with the generally low prevalence, the median PFU level in tissue samples was zero (upper quartile 107)

corresponding to agent level A0 (A3), that is, predominantly negative, but in some cases, low pathogen load. Only 5 (16·7%) of the 30 ultrafilter samples (corresponding to 100 L water per sample) collected from lake Stora Le were *A. astaci* positive. The detected spore concentration ranged from 0·05 to 9 spores L^{-1} in positive samples, and spores were detected at all subsites. The CPUE of signal crayfish were *c.* 5·7 (subsite D1), *c.* 4·4 (D2) and *c.* 0·6 (D3). At the subsite D3 with the lowest density of crayfish, *A. astaci* was detected in only one water sample (*c.* 0·8 spores L^{-1}) during the field season. None of the measured variables were able to predict the infrequent *A. astaci* spore content.

LAKE ØYMARKSJØEN, NORWAY (SITE E)

The overall observed prevalence of *A. astaci* in signal crayfish was 70%, but ranged from 30% to 100% during the season (Table S1, Supporting Information). The median PFU level in tissue samples was 82 (quartile 0, 473) corresponding to agent level A2 (A0, A3). A total of 27 (75%) of the 36 ultrafilter samples were *A. astaci* positive. The detected spore concentration ranged from 0·12 to 17 spores L^{-1}, in positive samples. The selected predictor variables in the nb-glm describing the spores L^{-1} were depth and weekly crayfish PFU. The overall, though insignificant trend (*P* = 0·056) indicated more spores at the lake bottom than at the surface (E1 and E2; Fig. 2). Even though the CPUE of the signal crayfish were *c.* 1·12 (subsite E1), *c.* 6·27 (subsite E2) and *c.* 0·79 (subsite E3), we observed no association between spore estimates and CPUE. There was a negative association (*P* = 0·004) between the spore concentration in the water (spores L^{-1}) and observed weekly crayfish pathogen load (mean PFU values in tissue samples).

OVERALL COMPARISONS

There was a significant positive association (*P* < 0·001) between the number of spores detected in a lake, the observed prevalence of *A. astaci*-positive signal crayfish in the corresponding population and the pathogen load (agent levels) in crayfish tissue samples (Fig. 3). There was also a difference (*P* < 0·001) in the amount of spores found in waters (lakes and farm) with latent carrier signal crayfish compared to the river undergoing a crayfish plague outbreak (Fig. 4, similar results are obtained when comparing only DF-based results, data not shown). Here, the water contained on average 43 times more spores than the waters hosting the latent carrier crayfish.

SENSITIVITY OF THE FILTERING TECHNIQUES

In our study design, we used two replicate samples for the DEUF and five replicate samples for the DF approach. With these settings, the model demonstrates that DEUF and DF were similar in sensitivity (Fig. 5a–c), provided that our assumptions about the low spore recovery during the DEUF protocol are correct. These assumptions (in addition to results from Smith & Hill 2009; Mull & Hill 2012) were supported by two observations during our study: (i) strong PCR inhibition in undiluted DNA templates obtained from DEUF made a 10-fold dilution of the templates mandatory, and (ii) the centrifugation of the suspension collected from the ultrafilters was in retrospect shown to be incomplete since some *A. astaci* spores remained in the discarded supernatant judged from positive qPCR results (qPCR results not shown). The simulation demonstrates that the approaches we used will reliable detect spores (≥ 95% detection probability) down
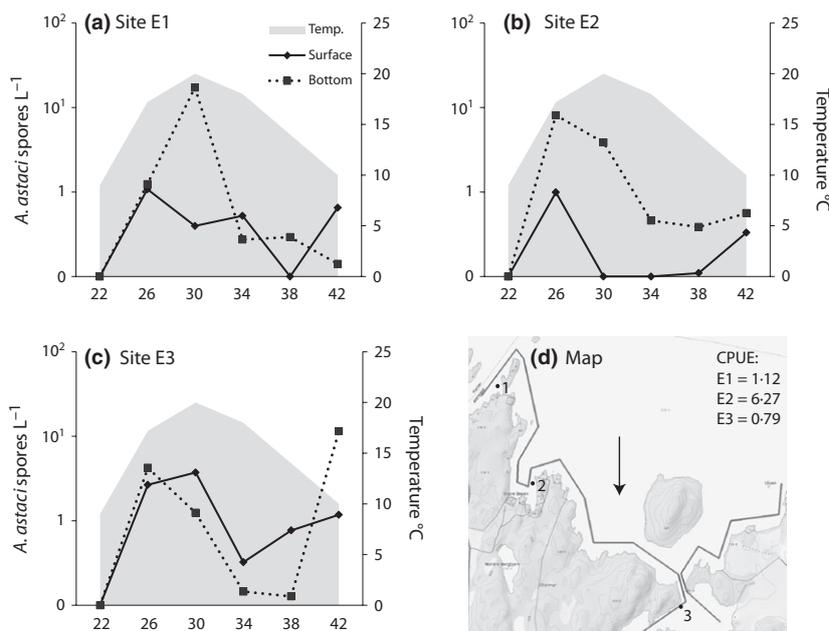

Fig. 2. (a–c) The estimated spore concentrations from each ultrafilter from Lake Øymarksjøen, Norway (site E) at each subsites (E1–E3) collected every fourth week during the summer 2011. Time of sampling is referred to as week numbers (May: 18–22, June: 23–26, July: 27–30, August: 31–34, September: 35–39, and October: 40–43). (d) Lake Øymarksjøen, with subsites (dots, E1–3) and the currently known distribution of signal crayfish (solid lines). Catch per unit effort (CPUE) is included.
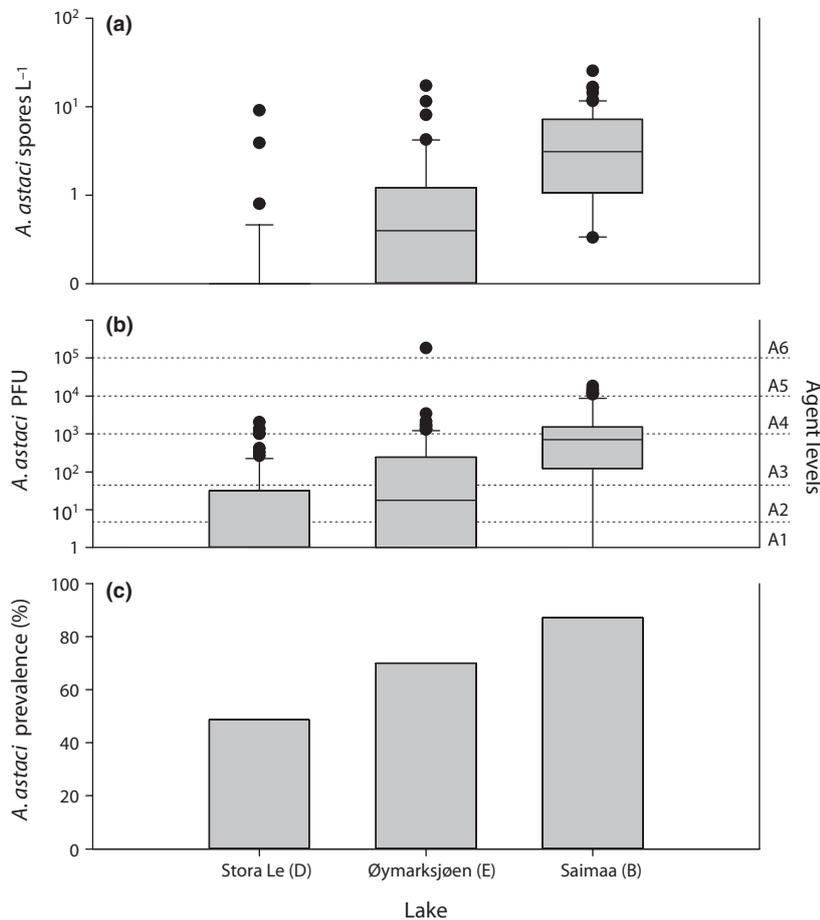
Fig. 3. A comparison of data from the three large lakes Stora Le (Sweden), Øymarksjøen (Norway) and Saimaa (Finland): (a) estimated *Aphanomyces astaci* spore concentration in water samples (spores L^{-1}; n = 30, 36 and 60 water samples from the respective lakes), (b) *A. astaci* PCR forming units (PFU) and corresponding agent level (Table S1, Supporting Information) in crayfish tissue samples (n = 70, 70 and 60 crayfish from the respective lakes), and (c) prevalence (%) of *A. astaci*-positive signal crayfish from the respective lakes (n = as in b).
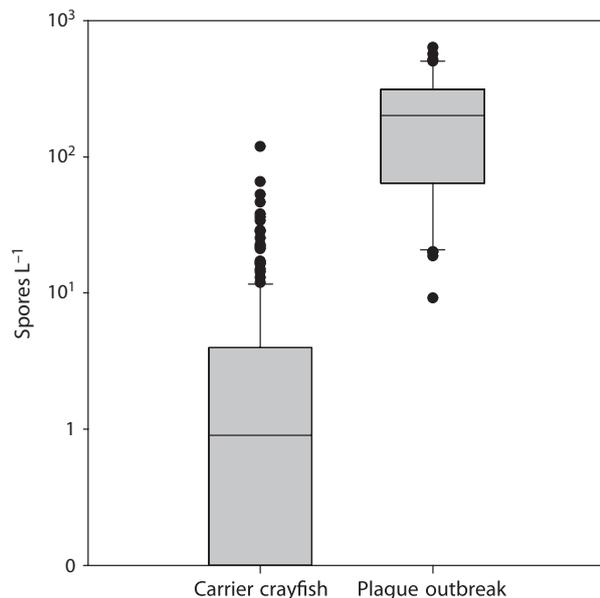

Fig. 4. Boxplot comparing the *Aphanomyces astaci* spore concentrations in (i) water bodies hosting signal crayfish (site A, B, D and E) and (ii) river hosting susceptible noble crayfish suffering from acute crayfish plague (site C). For (i), n = 138 depth filtration (DF) samples and 66 dead-end ultrafiltration (DEUF) samples representing in total 881 L water (DF) and 6600 L water (DEUF), respectively. For (ii), n = 38 DF samples representing 116 L in total.

to 1 spore L^{-1}, in some cases 0·1 spores L^{-1} (Fig. 5b,c) when the maximum number of replicate samples are used. Hypothetically, an optimization of the spore recovery from 50% to 90% from the suspension combined with elimination of the PCR inhibition would further yield a *c.* 10-fold increase in sensitivity of the DEUF approach (Fig. 5d), allowing reliable spore detection of 0·1 spores L^{-1}, and in some cases down to 0·01 spores L^{-1}.

Discussion

While Strand *et al.* (2011, 2012) demonstrated successful detection of *A. astaci* spores in water under laboratory and farm conditions, this study is the first to show a successful detection and quantification of the crayfish plague agent directly from large natural freshwater systems. The infrequent spore occurrence, often below 1 spore L^{-1} water, in lakes with signal crayfish compared to several hundred spores L^{-1} in the crayfish plague-affected river underlines the acute risk of disease transmission and spread during an outbreak situation, but also illustrates that even very low spore concentrations may pose a high transmission and infection risk judged from historical events (Bohman, Nordwall & Edsman 2006; Vrålstad *et al.* 2011). Additionally, there is also a chance for increased sporulation during moulting (Strand *et al.* 2012;
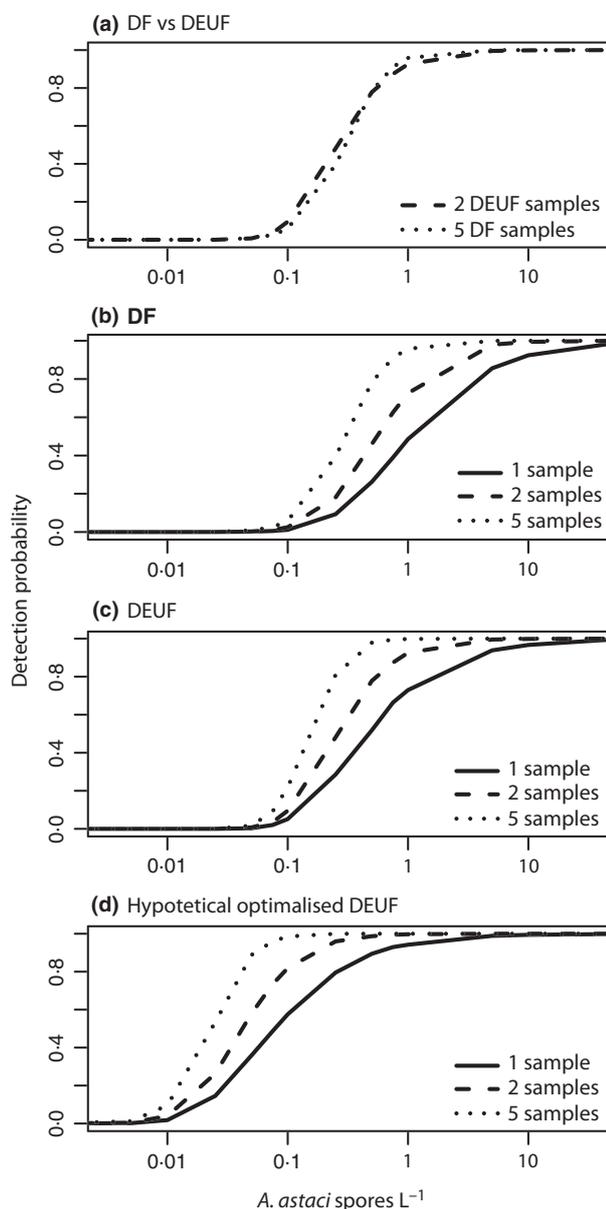
Fig. 5. The modelled sensitivity of the depth filtration (DF) and dead-end ultrafiltration (DEUF) approaches shown as the probability of detecting any spores for a given range of mean spore concentrations. The figures compare (a) the sensitivity of the DF (5 × 5 L samples) and DEUF (2 × 100 L samples) set-ups with the same number of replicate samples used in our surveys (5 DF and 2 DEUF, respectively), (b) the sensitivity of DF approach for one, two and five replicate samples, (c) the sensitivity of the DEUF approach for one, two and five replicate samples and (d) the sensitivity of a hypothetically optimized DEUF approach. According to the models, the sensitivity of the DF and DEUF approaches is equal when assuming suboptimal DEUF conditions, while the DEUF optimization in a hypothetical optimized protocol would yield a 10-fold increase in the sensitivity.

Svoboda *et al.* 2013), and this increase should be notable when synchronized moulting occur (Pratten 1980). Further, there are several reports where signal crayfish have suffered from acute crayfish plague (Edgerton *et al.* 2004) which is likely to result in increased sporulation (Strand

et al. 2012; Makkonen *et al.* 2013) also in waters hosting NICS.

The observed spatial and temporal differences in spore content in the waters, both within and between sites, suggest heterogeneous spore distribution and seasonal variations. In small aquaria systems, more *A. astaci* spores were detected in close vicinity to the crayfish at the tank bottom, than close to the water surface (Strand *et al.* 2012). We observed the same trend in the Norwegian lake, but the data is not clear enough to fully support this as a general pattern. This could partly be due to mixture of water at subsite E3 were the lake narrows and turns into a river. The water flow can also explain why we see the same level of spores at subsite E3, with very low crayfish densities, as the flow is from areas with higher densities. *Aphanomyces astaci* genotype group B associated with signal crayfish thrives best at temperatures ≤ 20 °C (Alderman & Polglase 1986; Diéguez-Uribeondo *et al.* 1995). Hence, the pronounced decline in spores in the Orivesi crayfish farm may be explained by the high water temperatures (above 22 °C).

The positive association between the *A. astaci* spore content in each lake, the observed *A. astaci* prevalence in the population, and the crayfish tissue pathogen load, suggests (not surprisingly) that high prevalence and high pathogen load generally leads to a higher spore density in the water. We can therefore predict large differences in spore concentrations among lakes hosting NICS since prevalence and *A. astaci* pathogen load varies substantially both between crayfish populations and species (Kozubíková *et al.* 2011; Vrålstad *et al.* 2011; Pârvulescu *et al.* 2012). The observed prevalence of *A. astaci*-positive crayfish in our study varied over the season within the same lake; however, the limited sample size (*n* = 10 per date) prevent clear conclusions. The observed negative association between spore concentration and weekly crayfish pathogen load of *A. astaci* in the Norwegian lake could also be a result of moulting events where recently moulted crayfish exoskeletons gave rise to increased *A. astaci* sporulation (Strand *et al.* 2012; Svoboda *et al.* 2013), while tissue analyses of the recently moulted crayfish would yield negative or low *A. astaci* PFU values. This emphasizes both that prevalence measures should take seasonal variation into account and that spore concentration cannot be assumed on the basis of prevalence measures from a single sampling event.

Next generation sequencing approaches (e.g. metagenomics and metabarcoding) of genetic material from any bulk sample can now study biodiversity based on environmental DNA (eDNA) across broad taxonomic scales (Lodge *et al.* 2012; Thomsen *et al.* 2012). Combined with qPCR approaches, this can provide revolutionary opportunities for species and biodiversity detection, estimates of relative abundance and population size, early detection of invasive and other harmful species including pathogens and surveillance of threatened species (Aw & Rose 2012; Lodge *et al.* 2012). However, the development of rapid,

sensitive and specific molecular tools often gains more attention than the upstream sampling processing (e.g. water filtration; Aw & Rose 2012). In studies from aquatic environments, the sample size varies greatly, between 10 mL to more than 200 L (Zinger, Gobet & Pommier 2012), and there is a knowledge gap on how field and laboratory protocols influence the detection of eDNA (Lodge *et al.* 2012). This will affect the likelihood of detection of a pathogen or the overall community representativeness. If small sample volumes are used, *A. astaci* and other pathogens with infrequent occurrence and/or heterogeneous distribution would be likely to go undetected or be excluded as singletons (DNA sequence detected once). This can to some extent be solved by increased sample volume by, for example, ultrafiltration (Lindquist *et al.* 2007; Gibson & Schwab 2011; Mull & Hill 2012).

The two filtering methods explored in this study (DF and DEUF) proved suitable for capturing infrequent *A. astaci* spores in large lakes. Our initial failure to detect spores in lakes hosting signal crayfish from ≤ 1 L water samples complies with our findings that more than 54% of the samples collected from signal crayfish lakes contained ≤ 1 spore L^{-1}. Regrettably, despite the much larger water volume sampled with the DEUF approach, the suboptimal conditions (i.e. low recovery; Smith & Hill 2009; Mull & Hill 2012; strong PCR inhibition, and spore loss during the centrifugation) made the DEUF and DF approaches equally sensitive according to our simulations. Improving the centrifugation step and inhibition constraints would alone yield at least a 10-fold increase in the DEUF sensitivity. Such optimizations, which need further research to identify and recommend, are therefore required before the DEUF approach can serve as a superior monitoring tool when using DNA technology for detection. The likelihood of spore detection increases with number of sample replicates (Fig. 5). This is particularly important to bear in mind for microbial diversity studies where samples collected only minutes and meters apart differ (Dolan & Stoeck 2011).

MANAGEMENT APPLICATIONS

We have successfully transferred the *A. astaci* detection and quantification approach from small controlled aquaria systems (Strand *et al.* 2012) to full-scale field conditions. For small systems with high crayfish densities (e.g. farms), or crayfish plague outbreaks, ≤ 5 L samples and the DF approach yielded good, quantifiable results. For larger lakes and water courses, or systems with low crayfish densities, the DF approach is still sufficient if replicates are used (e.g. 5×5 L samples). However, an optimized DEUF approach (*c.* 100 L) would drastically increase the probability of capturing low-prevalent spores. Methodological constraints probably led to a spore underestimation in our study, but the results illustrate nevertheless that an alien parasite with potentially lethal impact to the indigenous fauna can occur far below the detection limit of monitoring approaches that rely on small water volumes. Considering cost, the DF approach is less time-consuming and allows water sampling with standard, low-cost equipment and is thus a more affordable and feasible approach.

There are several potential applications of the method for conservation, management and diagnostic applications: (i) Early warning and targeted crayfish plague surveillance in natural habitats. (ii) A supplementary tool for assessing and evaluating pathogen status and/or habitat suitability, for example for crayfish re-stocking purposes or evaluating ark sites for conservation of threatened crayfish. (iii) More accurate risk assessments related to disease control and transmission risks than current standards, for example by water analyses in aquacultural systems (such as inlet/outlet water in farms) and prior to transportation of crayfish or fish from one location to another for stocking purposes. (iv) Water analysis of batches of many individuals (suspected carriers, vectors) in controlled tanks could be a good alternative to euthanizing multiple individuals for tissue analysis if the question is presence or absence of the pathogen (i.e. carrier or disease free, cf. Schrimpf *et al.* 2013b). However, the diagnosis of crayfish plague in susceptible species would still require tissue analysis (OIE 2012). Altogether, such measures could lead to substantially reduced transmission and spread of *A. astaci* within and between aquacultural systems and natural habitats. The methodological scope can also be transferred to other pathogen groups, or even widened to multiplex pathogen detection and eDNA monitoring of aquatic biodiversity (e.g. threatened and alien species).

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Pictures of the DF and DEUF systems.

Table S1. Prevalence and agent level of *A. astaci* in signal crayfish populations from four locations (A, B, D and E).