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Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments

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ABSTRACT: *Aphanomyces astaci*, a specialised parasite of North American freshwater crayfish, is the disease agent of crayfish plague that is lethal to European freshwater crayfish. The life cycle of *A. astaci* has been inferred from experimental laboratory studies, but less is known about its natural sustainability and ecology. To address such questions, tools for monitoring of *A. astaci* directly in aquatic environments are needed. Here, we present an approach for detecting and quantifying *A. astaci* directly from water samples using species-specific TaqMan® minor groove binder real-time PCR. Samples of a 10-fold dilution series from ~10^4 to ~1 spore of *A. astaci* were repeatedly tested, and reliable detection down to 1 spore was demonstrated. Further, to simulate real-life samples from natural water bodies, water samples from lakes of various water qualities were spiked with spores. The results demonstrated that co-extracted humic acids inhibit detection significantly. However, use of bovine serum albumin or the TaqMan® Environmental Master Mix largely removes this problem. The practical application of the approach was successfully demonstrated on real-life water samples from crayfish farms in Finland hosting infected North American signal crayfish *Pacifastacus leniusculus*. Direct monitoring of *A. astaci* from aquatic environments may find application in the management of wild noble crayfish *Astacus astacus* stocks, improved aquaculture practices and more targeted conservation actions. The approach will further facilitate studies of *A. astaci* spore dynamics during plague outbreaks and in carrier crayfish populations, which will broaden our knowledge of the biology of this devastating crayfish pathogen.

KEY WORDS: *Aphanomyces astaci* · Crayfish plague · Filtration · Molecular detection · Aquatic environments · Quantitative real-time PCR

INTRODUCTION

The crayfish plague agent *Aphanomyces astaci* (Saprolegniaceae, Oomycota) is lethal to freshwater crayfish that are not of North American origin (Unestam 1972, Söderhall & Cerenius 1999). Since *Aphanomyces astaci* was introduced to Europe in the late 19th century, indigenous crayfish populations have suffered heavy losses, and are still severely threatened (Holdich et al. 2009). Reproduction and spread of *Aphanomyces astaci* is accomplished by clonal, flagellated zoospores, which remain motile for up to 3 d and locate new crayfish hosts by chemotaxis. Failure to reach a crayfish host results in encystment of the zoospore (Söderhall & Cerenius 1999). The cysts may survive for 2 wk in distilled water (Unestam 1969), and 3 repeated zoospore emergences have been observed under laboratory conditions (Cerenius & Söderhall 1984), but spore via-

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bility and longevity may be prolonged at low temperatures (Unestam 1966). Prevailing evidence shows that *Aphanomyces astaci* is an obligate crayfish parasite not capable of surviving for a long period outside a crayfish host (Söderhall & Cerenius 1999). It is also generally accepted that *Aphanomyces astaci* causes 100% mortality in susceptible crayfish species. Consequently, *Aphanomyces astaci* may disappear relatively quickly from a freshwater environment in the absence of live crayfish hosts. This is supported by examples of successful reestablishment of European crayfish into water systems 1 yr after the original population was wiped out by crayfish plague (Söderhall & Cerenius 1999). However, there are examples of recurring outbreaks of crayfish plague after reintroductions of indigenous crayfish, despite years with no known crayfish in the water system. It is uncertain if this is due to reintroduction of crayfish plague, presence of unknown carrier crayfish or prolonged survival of *Aphanomyces astaci* in the environment (Westman 2000). The possibility that *Aphanomyces astaci* may have alternate hosts also needs to be thoroughly tested. In Norway, crayfish plague has reappeared several times in the same localities after a long fallow period before reintroduction of new crayfish (Vrålstad et al. 2006). In one case, illegally introduced signal crayfish *Pacifastacus leniusculus* infected with *Aphanomyces astaci* (Johnsen & Vrålstad 2009) explained the repeated crayfish-plague outbreaks and failure to reintroduce noble crayfish *Astacus astacus*. However, in other cases it is unknown whether the repeated events are due to undiscovered alien carrier crayfish or other complex factors (Vrålstad et al. 2006). In Finland, crayfish plague has apparently survived in the same water body for 10 to 25 yr and in some cases totally prevented the formation of new, utilisable crayfish populations (Jussila et al. 2008). It has also been shown under laboratory conditions that *Aphanomyces astaci* is able to complete all stages of its life cycle on fresh fish scales (Hall & Unestam 1980). Hence, many questions on the ecology of *Aphanomyces astaci* remain unanswered, and laboratory studies may poorly reflect the survival and persistence of *Aphanomyces astaci* under complex, natural conditions.

The development of PCR and real-time PCR methods for the specific detection of *Aphanomyces astaci* has accelerated the speed and significantly improved the accuracy of crayfish-plague diagnostics compared to culture-dependent approaches (Oidtmann et al. 2002, 2004, 2006, Hochwimmer et al. 2009, Vrålstad et al. 2009). Fast and reliable diagnostics of crayfish plague is important when considering countermeasures to prevent further spread of the disease. Furthermore, knowledge of the actual spore dynamics of *A. astaci* in lakes would be of great importance for justified assessments and decisions during and after plague outbreaks. Infection risk assessments, management of carrier crayfish populations, and evaluations of localities prior to reintroducing indigenous crayfish would also benefit from such knowledge. Studies of the prevalence of pathogenic viruses and microorganisms in water and the environment based on real-time PCR are rapidly expanding (Smith & Osborn 2009), but to date Oomycetes have been largely ignored. Here we present a real-time PCR-based approach for direct monitoring of *A. astaci* in aquatic environments. The practical performance is demonstrated on water samples from aquatic systems hosting infected signal crayfish.

MATERIALS AND METHODS

Preparation of spore units. In order to obtain quantitative units of *Aphanomyces astaci* for initial tests, spores were produced following the procedure described by Cerenius et al. (1988) with some minor modifications that included use of (1) smaller volumes (30 ml fluid in 50 ml falcon tubes), (2) a sterile scalpel for fragmentation of hyphae and (3) autoclaved (121°C, 15 min) water from a fish tank housing salmon parr instead of lake water. Two confirmed strains of *A. astaci* (VI03628 and VI04850, corresponding to EMBL/GenBank accession nos. AM947024 and FR694923 respectively) were used for spore production. These strains had previously been isolated from infected noble crayfish during crayfish plague outbreaks in the Halden water course (Norway) in 2005 and 2008, respectively. The strains are maintained on PG-1 (Söderhall et al. 1978) agar in the culture collection at the National Veterinary Institute (NVI), Norway. Unavoidably, the obtained spore suspension consisted of both spores and cysts, but both units contain one single genome and are in the present study referred to as spores or spore units. The spores produced were counted using a Brüker-Türk haemocytometer (W. Schreck, Hofheim/TS) on a light microscope at 10× magnification. A 10-fold dilution series using sterile distilled H_2O as diluent was prepared, starting at 10^4 spores 100 μl^{-1} down to 1 spore 100 μl^{-1}. The spore dilutions were used immediately after preparation to avoid settlement of cysts or formation of new cysts in the tubes.

Preparation of spiked water samples. In order to test the sensitivity of the chosen real-time PCR assay (Vrålstad et al. 2009) with reference to spore units of *Aphanomyces astaci* with and without a filtration step, the following samples were prepared. Five spore suspensions (the control samples) containing approximately 1, 10, 10^2, 10^3 and 10^4 spores in 100 µl were

transferred directly into 2 ml Eppendorf tubes. Further, 100 µl of the same spore suspensions were filtered (filter samples) on polycarbonate membrane filters (Millipore Isopore™, 3 µm pore size, 47 mm diameter) using the Microfil® Filtration System (Millipore). Each filter was loosely folded twice and transferred to a 2 ml Eppendorf tube using a sterile forceps. For each spore suspension, 10 replicate samples were prepared for both control and filter samples. The tubes were then frozen at –80°C awaiting DNA extraction.

To simulate real-life samples from natural water bodies, and to test the detectability of *Aphanomyces astaci* spores in such samples, water samples from 3 different lakes close to Oslo (Norway) with different water quality were collected. The 3 locations were Lutvann (oligotrophic), Årungen (eutrophic) and Sværsvann (dystrophic, stained by humic substances). A selection of limnological characteristics from these lakes is given in Table 1. Water samples of 1 l (surface water collected 13 May 2009) from each lake were spiked with 100 µl of 2 spore suspensions that contained approximately 10 and 10^3 spores, respectively (5 replicate samples per concentration, per lake). The water samples were filtered on membrane filters and processed as described in the preceding paragraph. For the controls, 100 µl of the same spore suspensions were added directly to 2 ml Eppendorf tubes (5 replicates for each control concentration).

Sampling and preparation of freshwater samples from a signal crayfish farm. To test the validity of the method, water samples and net haul samples were collected from ponds with signal crayfish of unknown carrier status at 3 crayfish farms in Finland (6 to 8 August 2009). Farm A consists of 1 indoor concrete tank and 2 outdoor ponds. The indoor tank is used as a holding tank for market-sized crayfish, collected from crayfish trappers from southern Finland, whereas under-market-sized crayfish are stocked in the outdoor ponds for growth. Farm B consists of 1 pond of roughly 1 hectare. The pond is used for recreational crayfish trapping for tourists. Farm C consists of 1 pond with a surface area of 400 m^2. Crayfish farm data, including estimates of the average number of crayfish individuals per area and water volume, are summarised in Table 2.

In all farms, water samples were collected at the bottom by submerging a Limnos water sampler (Hydro-Bios) to the bottom. Net haul samples were collected by a plankton net (10 µm mesh size), towed horizontally at approximately 0.5 to 1 m depth for 5 m. The number of water and net haul samples for each farm varied from 3 to 5 (details in 'Results'). Also, 10 signal crayfish were collected from each pond (except the indoor holding tank at Farm A) to determine their carrier status: soft abdominal cuticle, the proximal joint of 2 anterior walking legs and telson (according to Oidtmann et al. 2006) were tested with *Aphanomyces astaci*-specific real-time PCR according to Vrålstad et al. (2009).

DNA isolation. DNA was extracted from the samples with a modified version of the CTAB extraction protocol used by Vrålstad et al. (2009). The samples were initially frozen at –80°C in 2 ml Eppendorf tubes. One millilitre of pre-heated (65°C) CTAB buffer (20 g l^{-1} CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na_2EDTA) with 1% 2-mercaptoethanol was added to the filter samples, followed by vortexing for 10 to 15 s and subsequent freezing (–80°C for minimum 30 min). The samples were then heated to 65°C and 10 µl RNase A solution (10 mg ml^{-1}) was added, followed by mixing and incubation at 65°C for 30 min. Subsequently, 10 µl of Proteinase K (20 mg ml^{-1}) was added, followed by mixing and incubation at 65°C for 30 min. Chloroform (600 µl) was added and mixed by manual shaking for 15 s (this step dissolves the polycarbonate filter). The tubes were centrifuged (16 000 × *g*, 15 min), 800 µl of the aqueous phase was transferred to new tubes, 480 µl of isopropanol (stored at –20°C) was added and mixed in by inversion, and the sample was then incubated at room temperature for 15 min to precipitate the

Table 1. Selected limnological characteristics of the lakes used for simulation of real-life samples. Total P: total phosphorus in surface water; water colour: staining by humic matter compared to a standard solution; Pt: platinum

Lake	Trophic state	Total P (µg l^{-1})	Secchi depth (m)	Conductivity (mS m^{-1})	Water colour (mg Pt l^{-1})	pH
Lutvann	Oligotrophic	<5	>10	~6	<5	~7
Sværsvann	Dystrophic	18	<3	4.5	>60	<7
Årungen	Eutrophic	25–60	<1–2.5	15	~10–20	7–9.5

Table 2. *Pacifastacus leniusculus*. Summary data for the crayfish farms. Farm A consists of 3 locations: A1 (indoor tank), A2 (outdoor pond 1) and A3 (outdoor pond 2). Farms B and C are both outdoor ponds

Farm	Mean depth (m)	Area (m^2)	Volume (m^3)	No. of crayfish	Crayfish per unit area (ind. m^{-2})	Crayfish per unit volume (ind. m^{-3})
A1	0.35	40	14	1500	37.5	107.1
A2	1.3	1000	1300	2000	2.0	1.5
A3	1.5	1000	1500	4000	4.0	2.7
B	2.5	10000	25000	10000	1.0	0.4
C	1	400	400	1000	2.5	2.5

DNA. The tubes were centrifuged (16 000 × *g*, 15 min) and the supernatant carefully removed. The pellets were washed with 300 µl of 70% ethanol (stored at –20°C) and centrifuged (16 000 × *g*, 5 min). The supernatants were carefully removed and the pellets were dried for approximately 15 min using a vacuum dryer or heating block at 50°C. The pellets were then resuspended in sterile TE buffer. An environmental control (tube with sterile water left open on laboratory work bench during entire analytical process) and extraction blank control (sample tube subjected to DNA extraction process but without sample material) were included in the PCR to control for potential carry-over contamination (Vrålstad et al. 2009).

Real-time PCR. A TaqMan® minor groove binder (MGB) quantitative real-time PCR (qPCR) assay (Vrålstad et al. 2009) was used for the detection of *Aphanomyces astaci* in the sample DNAs. This assay targets a 59 bp sequence motif in the internal transcribed spacer 1 (ITS1) of the nuclear ribosomal gene cluster unique to *A. astaci*. Amplification and detection was performed on the Mx3005P qPCR system (Stratagene) in a total volume of 25 µl containing 12.5 µl Universal PCR Master Mix (Applied Biosystems), 500 nM of forward (AphAstITS-39; 5′-AAG GCT TGT GCT GGG ATG TT-3′) and reverse primers (AphAstITS-97; 5′-CTT CTT GCG AAA CCT TCT GCT A-3′), 200 nM MGB probe (AphaAstITS-60; 5′-6-FAM-TTC GGG ACG ACC C-MGB-NFQ-3′), 1.5 µl sterile Milli-Q water and 5 µl DNA template. Each DNA extract was always tested with an undiluted and a 10-fold diluted replicate. Also, 3 replicate qPCR runs were conducted per DNA extract from samples of the spore dilution series and the spiked natural water samples. The PCR programme consisted of an initial decontamination step of 2 min at 50°C to allow optimal uracil-DNA glycosylase (UNG) enzymatic activity, followed by 10 min at 95°C to activate the DNA polymerase, deactivate the UNG and denature the template DNA, and successively 50 cycles of 15 s at 95°C and 60 s at 58°C. The qPCR data was analysed using MxPro software v. 4.10 (Stratagene).

Quantification. In the present study we adopt the term PCR-forming units (PFU) as defined by Holst-Jensen & Berdal (2004) and also used in Vrålstad et al. (2009). A PFU corresponds to a single unit of a DNA target, in our case an ITS copy, that is amplifiable by PCR (i.e. not damaged or otherwise inhibited). Notably, the PFU may contain aggregated target sequence copies. In order to estimate the number of PFU from each spore concentration, 4 calibrant points from a standard series as described in Vrålstad et al. (2009) were included in each qPCR run in addition to the unknown samples. The limit of detection (LOD) and the limit of quantification (LOQ) for the specific *Aphanomyces astaci* TaqMan® MGB qPCR assay were previously estimated to be 5 and 50 PFU, respectively (Vrålstad et al. 2009). In the present study, all spore concentrations, both the control and filter samples, were detected and quantified based on the same detection and quantification limits. If the difference in the cycle threshold (C_t) value (ΔC_t) between the undiluted and 10× diluted DNA was <3, this was interpreted as evidence of inhibition of the qPCR for undiluted DNA. In the absence of inhibition, the qPCR results from the undiluted DNA extracts were used as the basis for PFU quantification. In the presence of inhibition, the qPCR results from the 10× diluted DNA extracts were used.

Diminishing amplification inhibition in real-time PCR. Humic substances, which are often present in water from freshwater lakes, are relatively hard to remove during DNA extraction and could inhibit enzyme activity during PCR amplification. Bovine serum albumin (BSA) has been shown to diminish the inhibitory effects of such substances (Kreader 1996). Due to clear inhibition in our spiked natural water samples, we tested if amplification inhibition was diminished with use of BSA in the qPCR reaction mix. All spiked water samples were re-tested with 4 different concentrations of BSA (5 mg in 0.25 ml; Fermentas) added to the reaction mix to final reaction concentrations of 0.1, 0.3, 0.5 and 1.0 µg μl^{-1}. Additionally, we also re-tested all spiked water samples and crayfish pond samples using the recently developed TaqMan® Environmental Master Mix (Applied Biosystems).

Statistics. To test if there were significant differences between the qPCR results of the control samples and filter samples, a linear mixed-effects model (including random slopes and intercepts for the replicates) was used in order to account for the dependence structure of the data. Based on the full model (i.e. including interaction term for the fixed effects), the random structure giving the smallest Akaike's information criterion (AIC) value was selected (Zuur et al. 2009). Results from both undiluted and diluted samples were included in the tests. Statistical tests were performed in the software R, v. 2.9.0 (R Development Core Team 2009), using the package *nlme* for mixed-effects modelling (Zuur et al. 2009).

RESULTS

Detection and quantification of *Aphanomyces astaci* spores

We detected spore concentrations down to approximately 1 spore $sample^{-1}$ (Fig. 1), which equals ~0.05 spore per PCR reaction since the DNA was eluted in

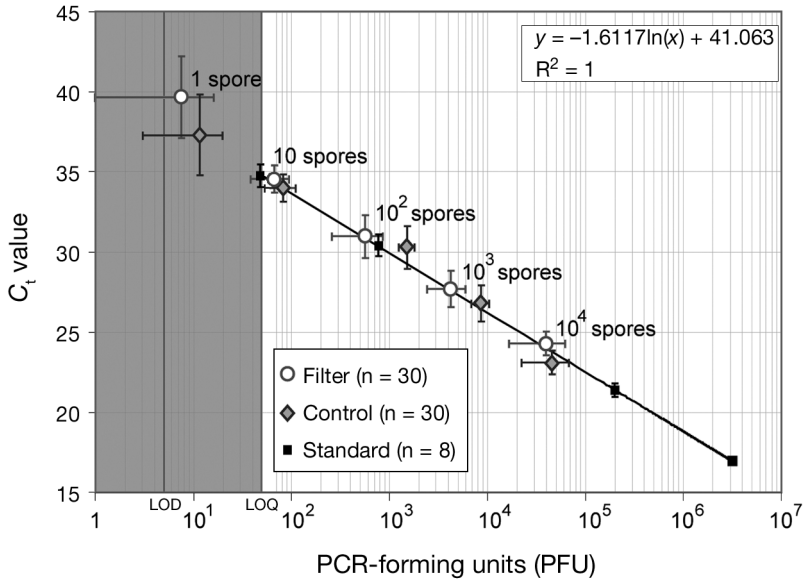

Fig. 1. *Aphanomyces astaci*. Mean cycle threshold (C_t) values (±SD) vs. mean PCR-forming units (PFU) (±SD) of the control and filter samples of the spore dilution series plotted against the standard curve consisting of 4 calibrant points. Shaded area is below the limit of quantification (LQR). Each point represents 10 replicates of each spore concentration and each replicate was run 3 times on quantitative PCR (qPCR). LOD = limit of detection

100 µl of TE buffer and only 5 µl of this genomic DNA sample was used in the subsequent qPCR reaction. Of single spore samples, 73.3% (filter) and 80% (controls) of the samples were amplified, of which 43.3% (filter) and 56.7% (controls) yielded a C_t value corresponding to >5 PFU (LOD) extrapolated from the standard curve. Further, of the 10 spore samples, 63.3% (filter) and 76.7% (control) were detected above the LOQ (≥50 PFU). Rough PFU estimates (based on the median and not the mean due to skewed data) per spore based on all control spore dilutions (1 to 10^4 spores) indicate that a single spore contains approximately ~138 PFU, although wide variability of the estimate is shown by the 10th and 90th percentiles (Table 3). Consequently, when using 0.05 units of the original DNA sample, the LOD corresponds approximately to ~0.7 spore and the LOQ approximately to ~7 spores in the original sample, respectively.

We observed a small but significant increase in the C_t value (0.84 ± 0.16 SE, $p < 0.0001$) of undiluted *Aphanomyces astaci* DNA for filter samples above LOQ compared to control samples (Fig. 2a; and the Supplement at www.int-res.com/articles/ suppl/d095p009_supp.pdf), but there was no significant linear interaction between spore concentration and filter treatment. However, the strength of the filter effect varied with spore concentration replicate (estimated SD of random slopes was 0.97). For diluted (×10) DNA, on the other hand, there was no significant difference in the C_t value between filtered and unfiltered samples (0.09 ± 0.24 SE, $p = 0.70$; Fig. 2a and the Supplement).

Table 3. *Aphanomyces astaci*. Estimated PCR-forming units (PFU) per spore, calculated as: spore dilution PFU estimate of control samples × 20 (as PCR reaction is based on 0.05 of whole DNA sample) / no. of spores. PCR reactions of 10 spore replicate samples in PCR triplicates

No. of spores in dilution	Median PFU estimate spore^{-1} (10th to 90th percentile)	No. of PCR reactions
1	149 (0–500)	30
10	177 (78–219)	30
10^2	346 (68–457)	30
10^3	160 (56–247)	30
10^4	92 (36–132)	30
Median total	138 (39–448)	150

Detection and quantification of *Aphanomyces astaci* spores in spiked natural water samples

The qPCR amplification showed clear signs of inhibition for all samples obtained from natural water bodies compared to controls. Neither 10 nor 1000 spores were

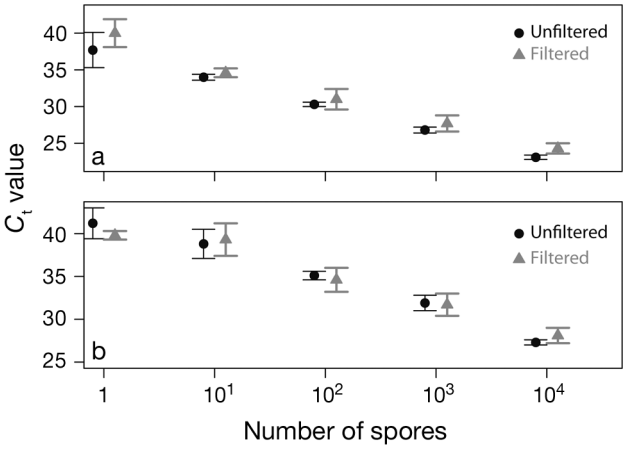

Fig. 2. *Aphanomyces astaci*. Mean cycle threshold (C_t) values (±SD) from control and filter samples of the spore dilution series, for (a) undiluted and (b) diluted DNA. For each replicate, the mean C_t value for 3 repeated measures was used. The observed difference in C_t values between filter and control samples was significant (0.84 ± 0.16 SE, $p < 0.0001$) for the undiluted *A. astaci* DNA samples, but not the 10× diluted DNA samples (0.09 ± 0.24 SE, $p = 0.70$)

possible to detect in the spiked natural water samples using the standard qPCR reagents (Fig. 3). BSA reduced the inhibition, but the needed concentration varied between the different water samples. For eutrophic water, 0.1 µg µl^{-1} BSA in the PCR reaction was sufficient to remove inhibition, while for the oligotrophic water, 0.3 µg µl^{-1} BSA efficiently removed inhibition. The inhibition was most prominent for humic-rich water samples, and even a BSA concentration of 0.5 µg µl^{-1} in the PCR reaction did not fully remove the inhibition. However, the TaqMan® Environmental Master Mix turned out to be more efficient than BSA, and removed close to 100% of the previously observed inhibition for all the different natural water samples (Fig. 3).

Detection and quantification of *Aphanomyces astaci* in crayfish and water from crayfish farms

From Farms A and B, 18 (90%) and 8 (89%) crayfish tested positive for *Aphanomyces astaci* in at least one of the tested tissue samples, respectively. In general, tissue samples of telson and walking legs yielded higher frequencies of positive results than samples of soft abdominal cuticle (Table 4). For Farm C, none of the 10 crayfish were positive for *A. astaci* in any of the tested tissues (Table 4). Results from the water and net haul samples are summarised in Table 5. From Farm A, the 3 samples from the indoor pond contained on average ~332 *A. astaci* genome units (assumed spores) per litre based on the assumption that 1 spore contains ~138 PFU. The outdoor ponds hosted a considerably lower density of spores. From the 2 outdoor ponds of Farm A, 50 and 40% of the samples in Ponds 1 and 2 were positive, respectively (above LOD), but with one exception below LOQ. Hence, <7 spores were detected in all positive samples apart from one, in which about 33 spores were estimated (Table 3). For Farm B, which consisted only of one large pond, <7 and ~18 *A. astaci* spores were detected in 2 respective water samples, while the remaining water samples and net haul samples were negative. All samples from Farm C were negative.

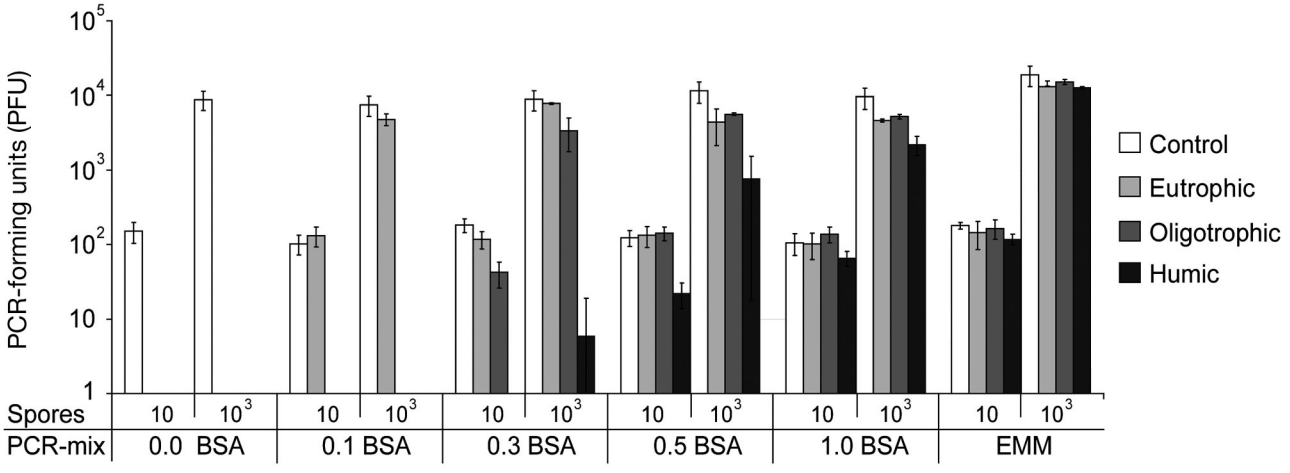

Fig. 3. *Aphanomyces astaci*. Quantitative PCR (qPCR) results in mean PCR-forming units (PFU) (±SD) of undiluted DNA from natural water samples spiked with 10 or 1000 spores l^{-1}. Different concentrations of bovine serum albumin (BSA) in a standard PCR mix and Environmental Master Mix (EMM) were compared. Five replicate samples were used per spore suspension and location, and 3 qPCR repeats per sample replicate

Table 4. *Aphanomyces astaci* infecting *Pacifastacus leniusculus*. Real-time PCR results testing for *A. astaci* DNA in crayfish from each farm or pond except Pond A1. Crayfish testing positive: *A. astaci* DNA detected in at least one of the tested tissue samples (n = 10 crayfish location^{-1}, except n = 9 for Farm B); PFU: PCR-forming units

Farm	Crayfish testing positive (%)	Cuticle		Walking leg		Telson	
		PFU (mean ± SD)	Positive samples (%)	PFU (mean ± SD)	Positive samples (%)	PFU (mean ± SD)	Positive samples (%)
A2	100	117 ± 309	40	552 ± 853	90	989 ± 1290	100
A3	80	13 ± 28	20	411 ± 911	50	90 ± 191	50
B	89	107 ± 289	22	48 ± 71	44	267 ± 534	89
C	0	0	0	0	0	0	0

Table 5. *Aphanomyces astaci*. Estimated PCR-forming units (PFU) values and spore numbers in analysed DNA from various water samples and net hauls collected from the 3 signal-crayfish farms in Finland. All positive samples were detected above the limit of detection. Sample ID: indicates Farm A (Pond A1, A2 or A3), B or C, and either a water sample (WS) collected by a 1 × 2 l water sampler at the bottom of each pond, or a water filtrate collected by a plankton net sampler, a 2 m net haul (NH) with 10 µm mesh size. Volume refers to the actual volume analysed (in many cases, filter clogging prevented filtration of the whole sample; subsamples for parts of the sampled volume were made and analysed separately, but here the results have been pooled; net hauls were collected in a 100 ml cup). Estimated no. of spores in sample based on median value of PFU estimate per spore (1 spore equals 138 PFU in 100 µl DNA sample)

Sample ID	Volume (l)	PFU in PCR reaction (5 µl DNA)	PFU in original DNA sample (100 µl DNA)	Rough spore estimate in sample
A1_WS1	1	1907	38140	276
A1_WS2	1	2782	55640	403
A1_WS3	1	2181	43620	316
A2_WS1	1	0	0	0
A2_WS2	1	<50	$<10^3$	<7
A2_NH1	0.1	0	0	0
A2_NH2	0.1	<50	$<10^3$	<7
A3_WS1	0.35	0	0	0
A3_WS2	0.6	<50	$<10^3$	<7
A3_WS3	0.3	<50	$<10^3$	<7
A3_WS4	0.3	0	0	0
A3_WS5	0.8	0	0	0
A3_NH1	0.1	<50	$<10^3$	<7
A3_NH2	0.1	0	0	0
A3_NH3	0.1	0	0	0
A3_NH4	0.1	0	0	0
A3_NH5	0.1	232	4641	33
B1_WS1	0.7	0	0	0
B1_WS2	0.8	0	0	0
B1_WS3	0.7	0	0	0
B1_WS4	0.4	<50	$<10^3$	<7
B1_WS5	0.6	126	2526	18
B1_NH1	0.1	0	0	0
B1_NH2	0.1	0	0	0
B1_NH3	0.1	0	0	0
B1_NH4	0.1	0	0	0
B1_NH5	0.1	0	0	0
C1_WS1	1.6	0	0	0
C1_WS2	1.5	0	0	0
C1_WS3	1.5	0	0	0
C1_WS4	1.5	0	0	0
C1_NH1	0.1	0	0	0
C1_NH2	0.1	0	0	0
C1_NH3	0.1	0	0	0

DISCUSSION

In the present study, we demonstrate that the capture of a single *Aphanomyces astaci* spore from a natural water body may be sufficient for subsequent molecular detection. Further, capturing 7 spores or more will allow for a crude quantification of the spore/genomic unit content of *A. astaci* in an individual water sample. A main source of uncertainty is the degree of heterogeneity of spore distribution in the water. In nature, heterogeneous distribution can be predicted both on a spatial and temporal scale. Hence, many samples of large water volumes must be analysed for an approximate estimate of the real-life situation of the spore dynamics in a lake. Nevertheless, our results demonstrate the sensitivity of the qPCR assay. Combined with optimised methods for concentrating large water volumes, the approach may allow for a specific pathogen search in natural environments even at very low spore levels.

Using spore suspensions of known spore content, we have demonstrated that the LOD (≤5 PFU) of the qPCR assay corresponds to the DNA content of approximately 0.7 spore, equivalent to 0.035 spore units per PCR reaction (given our experimental setup). Similarly, we have established that the LOQ (= 50 PFU) corresponds to the DNA content of approximately 7 spores, equivalent to 0.35 spore units per PCR. This is possible because the target DNA (ITS1) is present in multiple copies per genome (Long & Dawid 1980). Use of multi-copy target genes and connected spacer regions such as the nuclear ribosomal gene cluster provides a huge benefit over single and low-copy genes due to multiple-fold increased likelihood of detection. Assuming that the number of spores in our spore dilutions were correctly estimated, our results indicate that one *Aphanomyces astaci* spore includes on average ~138 PFU (amplifiable ITS copies). This should of course be taken as a very rough estimate that includes many sources of uncertainty (reflected in the percentile variation) including measuring errors in the initial spore counting, but illustrates nevertheless the power of ITS as target DNA when screening the environment for low concentrations of single-cell organisms. A possible way to further reduce the detection limit would be to up-concentrate the DNA by initial elution in smaller volumes and to use larger template DNA volumes per qPCR reaction.

The probe and primers used in the qPCR assay are highly specific to *Aphanomyces astaci* and have been tested against several Saprolegniaceae, including the closely related *A. invadans* and *A. frigidophilus* (Vrålstad et al. 2009). However, unknown diversity of putative closely related *Aphanomyces* spp. constitutes a challenge with regards to false positives. In a recent study (Wolinska et al. 2009), 3 unknown *Aphanomyces* taxa were discovered as parasites in *Daphnia* spp. in European lakes based on sequence information from nuclear ribosomal ITS region. These species grouped together with *A. invadans* and *A. frigidophilus*. It is therefore important to be cautious when interpreting

results based on real-time PCR data alone. In some cases, particularly if detection of *A. astaci* is unexpected, the use of additional confirmative tools such as conventional *A. astaci*-specific PCR described by Oidtmann et al. (2006) and DNA sequencing may be necessary to verify the results, provided there is a sufficient amount of target DNA in the sample. Future studies employing pyrosequencing of specifically amplified oomycete amplicons from natural water samples would indeed contribute to a better understanding of the natural diversity of *Aphanomyces* spp. and accompanying ITS-sequence variation that may challenge the present diagnostic assays for specific detection of *A. astaci*.

There are several potential sources of DNA loss during the processes of sampling, filtration and DNA extraction. Filtration could be expected to be a source of DNA loss if the captured spores were squeezed or burst open due to the vacuum pumping system, and consequently escaped as squeezed spores or free DNA through the filter pores. However, our results indicate that DNA is not lost during filtration or DNA extraction from filters, but that the filter itself may lead to minor inhibition of the qPCR detection of undiluted DNA samples.

Detection of microorganisms from laboratory water samples is usually straightforward, but natural waters contain several substances that potentially hamper molecular detection. Spiked natural water samples with low levels of *Aphanomyces astaci* spores simulating the real challenges we may face in the attempt to detect low levels of *A. astaci* in aquatic freshwater habitats confirmed that humic acids, which are commonly present in crayfish lakes, most severely inhibit PCR amplification. Use of BSA in the PCR mix, which has previously been reported to diminish humic acids inhibition (Kreader 1996, Reuter et al. 2009), was in the present study relatively efficient at a concentration of 0.5 µg µl^{-1}. However, the TaqMan® Environmental Master mix proved even more efficient and removed close to 100% of the observed qPCR inhibition from all water samples tested.

As the primary aim of the present study was to develop an approach for *Aphanomyces astaci* spore detection that would work in natural and semi-natural aquatic environments inhabited by crayfish, the successful detection of *A. astaci* spores directly from the 2 farms that hosted infected signal crayfish was a first breakthrough. Further, no positives were detected in water samples from the pond where no crayfish tested positive for *A. astaci* in any of the tested tissue samples. Even though 10 crayfish testing negative for *A. astaci* is insufficient to claim a negative carrier status of the signal crayfish population of Farm C, the apparent low prevalence of *A. astaci* compared to the other farms suggests that few or no spores of *A. astaci* would be present in the water. In this respect, the negative water samples provide indirect support for the specificity of the qPCR since no putative false positives were observed from the environmental samples of this farm. The highest levels of spores were detected in the indoor holding tank where large numbers of crayfish per water volume were kept in cages to be marketed later. The similar PFU values observed in these samples may indicate a relatively even distribution of spores, which could be expected due to thorough mixing of water in the indoor tank. On the other hand, the results from the outdoor ponds probably reflect a very low concentration of heterogeneously distributed spores. This could be explained by a more patchy distribution of crayfish, more water volume per individual and/or lower prevalence of *A. astaci* leading to heterogeneous spore release, fewer spores per water volume and consequently reduced likelihood of capturing spores in a water sample. Increased sample number or sample volume seems therefore essential for increased detection probability in systems with low spore density.

In the present study, clogging hampered the filtration of larger water volumes. Depending on the turbidity of the water, it was only possible to process water volumes from 0.3 to 1 l per membrane filter. Increased water volume per sample will require other filters or other filtration methods than applied here. We are therefore considering alternative filtration methods for further studies, e.g. ultrafiltration systems that have been described for filtration of microbes from drinking water (Hill et al. 2007, Lindquist et al. 2007, Francy et al. 2009). Provided one uses optimised methods for sampling and concentration of large water volumes, direct detection of *Aphanomyces astaci* from environmental freshwater samples will become a valuable tool both for monitoring purposes and basic research on the ecology and spore dynamics of *A. astaci* in natural freshwater systems. The approach may also find application in management of wild noble crayfish stocks, improved aquaculture practices and more targeted conservation actions. Still, serious obstacles must be overcome before detection of trace levels of *A. astaci* from large water volumes is realistic, e.g. for the purpose of unveiling small numbers of illegally introduced carrier crayfish, or declaring a locality free of crayfish-plague infection. These challenges are tightly linked to sampling and filtration capacity and technology, or alternatively more advanced spore-capturing technologies.

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