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Survey of the crayfish plague pathogen presence in the Netherlands reveals a new *Aphanomyces astaci* carrier

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ABSTRACT

North American crayfish species as hosts for the crayfish plague pathogen *Aphanomyces astaci* contribute to the decline of native European crayfish populations. At least six American crayfish species have been reported in the Netherlands but the presence of this pathogenic oomycete with substantial conservational impact has not yet been confirmed in the country. We evaluated *A. astaci* prevalence in Dutch populations of six alien crustaceans using species-specific quantitative PCR. These included three confirmed crayfish carriers (*Orconectes limosus, Pacifastacus leniusculus, Procambarus clarkii*), two recently introduced but yet unstudied crayfish (*Orconectes* cf. *virilis, Procambarus* cf. *acutus*), and a catadromous crab *Eriocheir sinensis*. Moderate levels of infection were observed in some populations of *O. limosus* and *P. leniusculus*. Positive results were also obtained for *E. sinensis* and two Dutch populations of *O.* cf. *virilis*. English population of the latter species was also found infected, confirming this taxon as another *A. astaci* carrier in European waters. In contrast, Dutch *P. clarkii* seem only sporadically infected, and the pathogen was not yet detected in *P.* cf. *acutus*. Our study is the first confirmation of crayfish plague infections in the Netherlands and demonstrates substantial variation in *A. astaci* prevalence among potential hosts within a single region, a pattern possibly linked to their introduction history and coexistence.

1. Introduction

The oomycete *Aphanomyces astaci* Schikora is the causative agent of the crayfish plague, a disease responsible for high mortalities of indigenous crayfish species throughout Europe (e.g., Alderman, 1996). It was suspected as early as in the 1960s that non-indigenous crayfish species (NICS) play a crucial role in the transmission of the crayfish plague pathogen to populations of native European crayfish (Unestam, 1969). All three North American crayfish invaders widely established in Europe, *Orconectes limosus* (Rafinesque), *Pacifastacus leniusculus* (Dana), and *Procambarus clarkii* (Girard), are confirmed carriers of *A. astaci* (Diéguez-Uribeondo and Söderhäll, 1993; Unestam, 1972; Vey et al., 1983). These species had been imported to Europe before 1975 for stocking purposes and have become widespread since then (Holdich et al., 2009; Kouba et al., 2014).

At least seven other crayfish species of North American and Australasian origin have become established in Europe more recently, mainly thanks to introductions from aquarium trade and aquaculture (Holdich et al., 2009). Five of these "new NICS" are of North American origin, and thus potential carriers of *A. astaci* (see Oidtmann, 2012; Unestam, 1972, 1969). However, it has been shown that the prevalence of *A. astaci* may substantially vary among species, regions, and even local populations (e.g., Filipová et al., 2013; Kozubíková et al., 2011a; Schrimpf et al., 2013a). Thus, the potential to spread *A. astaci* cannot be assessed unless a particular species (population) is tested for the presence of the pathogen. So far, only one of the new NICS, the calico crayfish *Orconectes immunis* (Hagen), has been confirmed as a vector of this pathogen (Filipová et al., 2013; Schrimpf et al., 2013b). Nevertheless, these findings highlight the potential of other newly introduced North American crayfish species to spread the crayfish plague agent.

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To date, seven non-indigenous crayfish species have been reported in the Netherlands (although the taxonomic status of some of them is not entirely clear; see Filipová et al., 2010, 2011). These include: narrow-clawed crayfish *Astacus leptodactylus* (first reported in 1982), spiny-cheek crayfish *Orconectes limosus* (1973), virile crayfish *O.* cf. *virilis* (2006), signal crayfish *Pacifastacus leniusculus* (2005), white river crayfish *P.* cf. *acutus* (2006), red swamp crayfish *Procambarus clarkii* (1989), and marbled crayfish *P. fallax* f. *virginalis* (2006) (Adema, 1989, 1982; Geelen, 1978, 1975; Geelen and Oomen, 1973; Soes and van Eekelen, 2006; Soes and Koese, 2010). While *A. leptodactylus* originates from Eastern Europe, the other six alien crayfish species found in the Netherlands are of North American origin. Although the present status of the marbled crayfish population is unclear (Soes and Koese, 2010), the country still harbors one of the highest numbers of potential crayfish plague carriers in Europe (see Kouba et al., 2014). Moreover, since the early 1930s Dutch waters have been invaded by the Chinese mitten crab *Eriocheir sinensis* (Herborg et al., 2003; Kamps, 1937), which can also get infected by the pathogen from carrier crayfish (Svoboda et al., 2014).

In contrast with apparently thriving alien crustaceans, Dutch populations of indigenous noble crayfish *Astacus astacus* have disappeared at an alarming rate since the second half of the twentieth century. Whereas during the period from 1660 to 1947, 38 Dutch localities were still inhabited by *A. astacus*, their number gradually decreased over time (Geelen, 1978), and presently only one residing population remains (Ottburg and Roessink, 2012).

The presence of *A. astaci* in the Netherlands was never officially confirmed although epizootics of crayfish plague were implicated as one of the major reasons for the decline of native crayfish in the Netherlands. For example, this disease was the presumed cause of the mass mortality of some of the last Dutch populations of *A. astacus* in the Roosendaalse Brook in 2001 (Niewold, 2002), since when only a single population in the Netherlands remains in an isolated pond near Arnhem. Infection by this pathogen has only been studied for one Dutch population of *A. leptodactylus* so far, and the few screened individuals tested negative (Roessink and Ottburg, 2012). As a reintroduction program aiming to increase the number of noble crayfish populations in the Netherlands has been recently launched (Ottburg and Roessink, 2012), knowledge on the distribution of the crayfish plague pathogen is of paramount importance for its success.

In the present study, we screened populations of all five well established North American alien crayfish species as well as one population of the Chinese mitten crab with the OIE-recommended (Oidtmann, 2012) molecular diagnostic methods to confirm the infection by *A. astaci*. Based on experience from other European countries, we expected a widespread presence of *A. astaci* in populations of the well-known and common *A. astaci* carriers (*O. limosus*, *P. leniusculus, P. clarkii*). We also hypothesized that individuals of *E. sinensis* would test positive, since they are in contact with North American crayfish in Dutch waters, and thus can get infected. For the first time, we also provide results of testing of two recently introduced crayfish taxa, *Orconectes* cf. *virilis* and *Procambarus* cf. *acutus*, for which no data on *A. astaci* infections were previously available. We assumed that due to their North American origin, they may also host *A. astaci* in European waters.

2. Materials and methods

2.1. Sampling and DNA extraction

To evaluate the presence of *A. astaci* in Dutch waters, populations of five North American alien crayfish (spiny-cheek crayfish *Orconectes limosus*, virile crayfish *Orconectes* cf. *virilis*, signal crayfish *Pacifastacus leniusculus*, white-river crayfish *Procambarus* cf. *acutus*, and red swamp crayfish *Procambarus clarkii*) and one Asian crab species that gets into contact with potential *A. astaci* carriers in Dutch freshwaters (Chinese mitten crab *Eriocheir sinensis*) were sampled. The approximate locations of the sampled populations are presented in Fig. 1. Their exact position, sampling details, and number of individuals sampled per population are summarized in Table 1.

Sample storage, processing and DNA isolation slightly differed as samples from the involved localities were processed independently in two laboratories. Selected samples of all five crayfish taxa were analyzed at the Central Veterinary Institute in Lelystad, the Netherlands (CVI). In parallel, other samples of four of these taxa (all but *P. leniusculus*) and samples of *E. sinensis* were analyzed at the Department of Ecology, Charles University in Prague, Czech Republic (CUNI). To confirm correct detection of the pathogen and to compare the quantitative results obtained in the two laboratories, a selection of DNA isolates was analyzed both at CUNI and at CVI.

Upon sampling, specimens were stored in plastic bottles filled with 96% ethanol (CUNI), or frozen and stored at −20 °C (CVI). We dissected either soft abdominal cuticle, any melanization on the body visible by naked eye, and pieces of two uropods (CUNI) or exclusively soft abdominal cuticle (CVI) from each crayfish individual. From crab specimens we used soft cuticle from telson and abdomen, 4 joints from chelipeds, second pair of maxillipeds, and any melanized wounds after a pereiopod loss. Dissection tools were cleaned with UV-light and sodium hydroxide, or with hydrogen peroxide and flame sterilization after dissection of each individual to prevent cross-contamination. The dissected tissues were pooled together in order to obtain one DNA isolate for each specimen.

Prior to DNA extraction, the tissues were mechanically disrupted and homogenized. Grinding in sterile mortars with liquid nitrogen was used at CUNI. At CVI, the tissues were homogenized with TeSeE PRECESS 24 homogenizer (BioRad) in IDEXX tissue

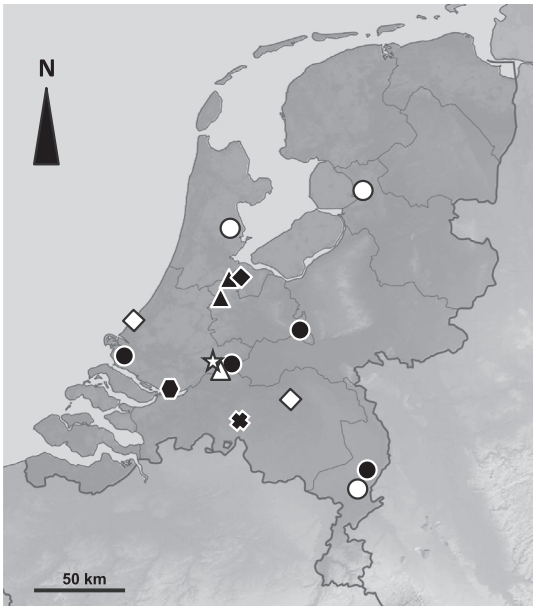

Fig. 1. Map of the Netherlands with approximate locations of analyzed populations of *O. limosus* (circle), *O.* cf. *virilis* (triangle), *P. leniusculus* (cross), *P. acutus* (star), *P. clarkii* (diamond) and *E. sinensis* (hexagon). Populations in which *A. astaci* infection was detected are indicated by black shapes, those without *A. astaci* detection by white shapes. In cases where sampled populations are in close vicinity to each other, only one location is marked in the map.

Table 1
Results of *Aphanomyces astaci* detection in populations of five North American crayfish and one Asian crab species occurring in the Netherlands. For positive detections, semi-quantitative agent levels are provided.

Sp.	Sampling site	River basin	Type of water body	Coordinates		Month of sampling	Individuals tested	*A. astaci* infected	Prevalence (95% CI)	Agent level			
				Latitude (N)	Longitude (E)					A2	A3	A4	A5
Orconectes limosus													
	Brielle	Rhine	Ditch	51°54′00″	4°10′00″	September 2012	6	1	16% (0.4–64%)	1			
	Gorinchem[a]	Rhine	River	51°50′08″	4°56′07″	May 2012	19	5	26% (9–51%)	1	3	1	
	Gouwzee	Rhine	Lake	52°26′21″	5°03′24″	February 2013	6	–	0% (0–58%)				
	Meuse	Meuse	River	51°17′32″	6°04′05″	October 2013	13	3	23% (5–54%)	2	1		
	Roermond	Meuse	River	51°11′23″	5°58′52″	June–August 2012	10	6	60% (26–88%)	3	3		
	Wageningen[a]	Rhine	Canal	51°57′58″	5°37′05″	October 2012	5	4	80% (28–99%)	1	3		
	Zwarte meer	Rhine	Lake	52°38′36″	6°00′17″	February 2013	7	–	0% (0–53%)				
Orconectes virilis													
	Boven-Hardinxveld[b]	Rhine	Ditch	51°50′01″	4°54′23″	May & December 2012	2 + 1	–	0% (0–80%)				
	Kanis[a]	Rhine	Canal	52°08′21″	4°53′35″	October 2012	7	4	57% (18–90%)	3		1	
	Oukoop[a]	Rhine	Ditch	52°13′07″	4°58′57″	September 2012	12	7	58% (28–85%)	4	3		
Pacifastacus leniusculus													
	Tilburg	Meuse	Brook	51°31′04″	5°04′43″	June 2012	5	4	80% (28–99%)	3	1		
Procambarus acutus													
	Alblasserwaard	Rhine	Ditch	51°52′01″	4°53′07″	September 2012	13	–	0% (0–34%)				
	Boven-Hardinxveld[a]	Rhine	Ditch	51°50′01″	4°54′23″	May 2012 & March 2013	20 + 20	–	0% (0–13%)				
	Giessenburg [a]	Rhine	Ditch	51°51′26″	4°54′18″	May 2012	20	–	0% (0–24%)				
Procambarus clarkii													
	Den Haag[a]	Rhine	Ditch	52°04′47″	4°15′34″	August 2012	20	–	0% (0–24%)				
	Schijndel[a]	Meuse	Ditch	51°38′09″	5°27′06″	October 2012	20	–	0% (0–24%)				
	'Terra Nova'	Rhine	Lake	52°13′09″	5°02′13″	October 2012	10	1	10% (0–45%)	1			
Eriocheir sinensis													
	Hollandsch Diep[a]	Rhine	River	51°41′55″	4°28′30″	September & November 2012	29	5	17% (6–36%)	2	1	1	1

[a] Populations analyzed at CUNI.
[b] Independently in both laboratories.

disruption tubes equipped with ceramic beads for 2 × 45 s, followed by freezing at −20 °C and a second disruption step of 2 × 45 s of the still frozen material.

Up to 40 mg of the homogenized tissues of each individual was used to obtain the DNA isolates with the DNeasy tissue kit (Qiagen) at both laboratories. A DNA extraction control (an Eppendorf tube containing Milli-Q water, treated as other sample-containing tubes) was prepared during each isolation batch to control for potential cross-contamination among samples. One control was included for every 10 samples. These remained negative in all cases. The DNA isolates were stored at −20 °C.

2.2. Aphanomyces astaci detection

To test for the presence of *A. astaci* DNA, the quantitative PCR assay was performed as described by Vrålstad et al. (2009), with minor modifications that differed at the two laboratories. At CVI, the qPCR reaction was carried out with 1× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), 1.25 units of Uracil-DNA Glycosylase (New England BioLabs) were added per reaction to prevent carry-over contamination, and the final volume was 20 µl per reaction. The qPCR was carried out in an AB 7500 with fast block (Applied Biosystems) according to the program: 37 °C for 10 min, 95 °C for 10 min followed by 50 cycles of 95 °C for 3 s and 58 °C for 30 s. At CUNI, the qPCR was performed on an iQ5 (Bio-Rad), the TaqMan Environmental Master Mix (Applied Biosystems) was used to reduce the potential PCR inhibition (see Strand et al., 2011) and annealing temperature was increased (from 58 to 62 °C) while synthesis time was decreased (from 60 to 30 s) to further increase the assay specificity (Strand, 2013).

At both laboratories, undiluted and 10× diluted original DNA isolates, a DNA extraction control, and a PCR blank control were included in each run. The number of PCR-forming units (PFU) added to each reaction was calculated using a standard curve, whose construction differed in detail between the two laboratories. At CVI, the method described in Kušar et al. (2013) was followed: dilution series of *A. astaci* DNA was prepared and analyzed in triplicates in three qPCR runs to give one standard curve, which was used to calculate the numbers of PFU in all samples according to their qPCR results. More details about this procedure are given as supporting information (Table S1, Fig. S1 in the Electronic Supplementary Material). At CUNI, the protocol published by Vrålstad et al. (2009) was followed; four *A. astaci* calibrants were prepared and used in every qPCR run to generate a standard curve. The number of PFU in an original DNA isolate was calculated according to Kozubíková et al. (2011a). Eventually, the quantitative results in PFU (obtained in either laboratory) were translated into more comprehensible and more robust semi-quantitative agent levels (A0–A7; Kozubíková et al., 2011a; Vrålstad et al., 2009). A test performed with identical DNA isolates in both laboratories indeed confirmed that both approaches gave comparable results despite the methodological differences described above. The isolates which did not contain any *A. astaci* according to CVI tested negative (agent level A0) also at CUNI. The quantitative data obtained in the two laboratories for other isolates (agent level A2–A7) mostly corresponded as well, resulting in different (but neighboring) agent levels only occasionally for samples containing *A. astaci* DNA in concentrations close to the limits of detection and quantification (see more details in Electronic Supplementary Material).

Since we tested for the presence of *A. astaci* in our samples using molecular methods only, the results could have been biased if DNA had not been isolated in sufficient quality and quantity from tested tissues (e.g., due to poor quality of the samples, or handling mistakes during DNA isolation). Thus, a few DNA isolates of apparently insufficient quality were excluded from the results to minimize the possible bias caused by false negatives, i.e., isolates from tissues parasitized by the pathogen but resulting in no detection of *A. astaci* DNA in the analyses. At CUNI, DNA concentration of all DNA isolates was estimated with the Nanodrop 1000

Spectrophotometer (Thermo Fisher Scientific). Two isolates out of 147 with outlying absorbance ratios (at 260/230 nm and 260/280 nm) indicating substantial presence of contaminants were excluded. Furthermore, the difference in PFU in undiluted and 10× diluted DNA isolates was used to check for potential inhibition of qPCR (for details, see Kozubíková et al., 2011a); no such inhibition was observed. At CVI, the Eukaryotic 18S rRNA Endogenous Control kit (Applied Biosystems) was used according to the manufacturer's protocol to check the integrity of the isolated crayfish DNA. Out of 75 analyzed samples, four were omitted from the dataset that yielded high Ct values (Ct > 26), i.e., suggesting low concentration or quality of host DNA, and at the same time tested negative for *A. astaci*.

The confirmation of *A. astaci* DNA in samples from representative infected populations that yielded positive qPCR results proceeded with sequencing of a 569 bp long amplicons including parts of internal transcribed spacers (ITS) 1 and 2 and 5.8S rDNA according to Oidtmann et al. (2006), as recommended by OIE (Oidtmann, 2012). As the conventional PCR is less sensitive than the qPCR approach (Kozubíková et al., 2011b; Tuffs and Oidtmann, 2011), the ITS sequences were obtained from infected individuals with agent levels A3 and higher according to qPCR analysis. The PCR products of these *A. astaci*-positive isolates were purified with ethanol precipitation and sequenced in both directions on the ABi 3130xl Genetic Analyzer (Applied Biosystems). Resulting sequences were compared to publicly available sequences of *A. astaci*, and the representative ones were deposited to GenBank (KF944440–KF944443, KJ710432–KJ710434).

To estimate prevalence in studied populations, we calculated 95% confidence intervals for the prevalence values obtained from the number of *A. astaci*-positive and total number of tested samples per population. This was conducted as in Filipová et al. (2013), using the function "epi.conf" from the library epiR (Stevenson et al., 2013) for R v. 3.0 (R Core Team, 2013).

3. Results

The presence of *Aphanomyces astaci* was detected in populations of four North American crayfish and one Asian crab species present in the Netherlands (*O. limosus*, *O.* cf. *virilis*, *P. leniusculus*, *P. clarkii* and *E. sinensis;* Table 1). Out of 216 examined crayfish and 29 crab individuals, 35 crayfish and five crabs tested positive for the pathogen. The isolates positive for *A. astaci* reached low (A2) to high (A5) agent levels (Table 1).

For confirmation of *A. astaci* infections, we obtained eight ITS sequences from representative populations of four host taxa: *O. limosus* (Gorinchem, Meuse, Roermond, and Wageningen), *O.* cf. *virilis* (Kanis and Oukoop), *P. leniusculus* (Tilburg), and *E. sinensis* (Hollandsch Diep). These were all identical to the *A. astaci* reference sequences available in GenBank. From a single apparently infected specimen of *P. clarkii*, no ITS sequence was obtained, presumably due to low level of pathogen infection.

The pathogen prevalence in all studied populations was highly variable, ranging from 0% to 80%. However, as the wide confidence intervals for the prevalence estimates indicate, the lack of detection in most populations (especially those with relatively low numbers of individuals analyzed) cannot be considered an evidence of absence of the crayfish plague pathogen in these populations.

Individuals infected with *A. astaci* were detected in four out of seven tested populations of *O. limosus*, the most widespread and common alien crayfish in the Netherlands (Table 1). In these populations, the prevalence ranged from moderate (16–25%) to high (80%). Moreover, high prevalence (80%) of *A. astaci*-positive crayfish was observed in the analyzed population of *P. leniusculus*. In contrast, in *P. clarkii,* the second most widespread alien crayfish in the Netherlands, only one individual out of 50 analyzed (from three populations) tested positive for the pathogen, with a very low agent level (A2).

Contrasting patterns of crayfish plague prevalence were also observed in populations of the recently introduced crayfish species, *P.* cf. *acutus* and *O.* cf. *virilis.* Despite an extensive sampling (73 individuals analyzed from three sampling sites) no pathogen was detected in specimens of the former, whereas moderate prevalence (57–58%) of *A. astaci* infection was detected in two out of three populations of the latter.

4. Discussion

The Netherlands harbors one of the highest diversity of nonindigenous crayfish in Europe, comprising established populations of six North American and one Eastern European species (Kouba et al., 2014). The first study focusing on the presence of *A. astaci* in Dutch crayfish (Roessink and Ottburg, 2012) did not detect any infection in *A. leptodactylus* population. Our study, however, unambiguously revealed the presence of the crayfish plague pathogen in the Netherlands in its natural hosts, i.e., North American crayfish species, and additionally in the Chinese mitten crab.

Relatively high prevalence of infection was repeatedly detected in *O. limosus,* the most widespread nonindigenous crayfish in Dutch waters (Soes and Koese, 2010). This species thus serves as an important *A. astaci* reservoir in the Netherlands. In addition, high prevalence was also observed in the studied *P. leniusculus* population. Although the range of this host species is still restricted to only two water bodies near the eastern and southern borders of the country (Soes and Koese, 2010), its further expansion is likely and the species may therefore contribute to spread of the crayfish plague pathogen in Dutch waters. Moreover, *A. astaci* was also detected in the tissues of some *E. sinensis*, a migratory crab species capable of covering long distances (Herborg et al., 2003; Kamps, 1937). The crabs likely acquired the infection from coexisting *O. limosus,* widespread in the rivers Rhine and Meuse (Soes and Koese, 2010), whose populations in these basins are infected by *A. astaci* (Fig. 1, Table 1).

Aphanomyces astaci infections were also detected for the first time in Dutch *Orconectes* cf. *virilis*, a recent North American invader shown to represent a distinct clade within the virile crayfish species complex (Filipová et al., 2010). The virile crayfish is the second new NICS found in European waters testing positive for this pathogen's presence, after *Orconectes immunis* in Germany and France (Filipová et al., 2013; Schrimpf et al., 2013b). To date, *O.* cf. *virilis* has a restricted distribution in Europe, limited to the Netherlands and the United Kingdom, and molecular analyses revealed that both populations belong to the same phylogenetic lineage (Filipová et al., 2010). This suggests a common introduction pathway for both populations. Interestingly, an independently conducted analysis (B. Oidtmann and S. Nutbeam-Tuffs, unpubl. data) confirmed that the English population of this species has also a high prevalence of *A. astaci.* Out of 21 specimens sampled in October 2009 from the River Lee (the Thames catchment) and analyzed with the same ITS-based qPCR detection method (Vrålstad et al., 2009), 18 individuals (86%) tested positive.

The identity of *A. astaci* infections in the virile crayfish deserves further consideration. Four different *A. astaci* genotype groups, associated with different host species, are known so far (Diéguez-Uribeondo et al., 1995; Huang et al., 1994; Kozubíková et al., 2011b) but it is not unlikely that additional *A. astaci* strains, differing in such properties as virulence (Jussila et al., 2011; Makkonen et al., 2014; Viljamaa-Dirks et al., 2011) or climate requirements (Diéguez-Uribeondo et al., 1995; Rezinciuc et al., 2013), may be

introduced with new host taxa. We consider likely that the virile crayfish had been already infected prior to its introduction. Similarly as for *O. immunis* (Schrimpf et al., 2013b), we may speculate that in such case, European populations of *O.* cf. *virilis* might carry their own specific strain of *A. astaci*. However, we cannot also exclude independent horizontal transmission of the pathogen from another infected species after establishment of the virile crayfish in both the Netherlands and the United Kingdom.

The confirmation that *O.* cf. *virilis* is another *A. astaci* carrier supports the assumption that North American crayfish species in general have the potential to carry latent *A. astaci* infections (Oidtmann, 2012). This is further supported by the observation that crayfish spread through the pet trade may occasionally be infected by *A. astaci* (A. Mrugała et al., unpubl. data). Interestingly, unlike for *Orconectes* spp. and *P. leniusculus*, only one very weakly infected individual was observed for *Procambarus clarkii,* an important *A. astaci* host elsewhere in Europe (Aquiloni et al., 2011; Rezinciuc et al., 2013), and no *A. astaci* infection was observed in examined Dutch specimens of *P.* cf. *acutus*. The three sampling sites of *P.* cf. *acutus* are geographically close to each other, likely originating from a single original source. The apparent absence (or very low prevalence) of the pathogen in studied population of this taxon might thus result from a founder effect. That would be in accordance with several studies investigating the presence of *A. astaci* infections in established populations of North American crayfish across Europe, which also did not detect the pathogen in at least some of the studied populations (e.g., Schrimpf et al., 2013a; Skov et al., 2011; but see Kozubíková et al., 2011a). In particular, the results of Schrimpf et al. (2013a) reveal that some European populations of these crayfish may be free of this pathogen.

The variability in *A. astaci* prevalence in populations of its natural carriers has been explained by several factors, including: age and size of sampled individuals (Vrålstad et al., 2011), temporal fluctuations in pathogen presence (Matasová et al., 2011), and the type of the water body inhabited by crayfish (Kozubíková et al., 2009). Additionally, pathogen prevalence may also be shaped by introduction history, i.e., infection levels and life-stage of founder individuals (Kozubíková et al., 2009; Torchin et al., 2003). In this context, however, it remains open whether the contrasting patterns of *A. astaci* prevalence in Dutch *Orconectes* and *Procambarus* populations are linked to their different origin and introduction pathways or whether other factors mentioned above played a key role.

It is worth attention that *P.* cf. *acutus* in the Netherlands gets into contact with several other North American crayfish (*O. limosus*, *O.* cf. *virilis*, and *P. clarkii*). As Dutch *Orconectes* spp. are frequently infected by *A. astaci*, pathogen transmission from these species to *P.* cf. *acutus* may be eventually expected. However, it is possible that horizontal transmission of a particular *A. astaci* genotype between different North American host taxa is limited by host-pathogen incompatibilities.

In the future, more detailed sampling, including in particular locations where multiple potential *A. astaci* carriers coexist, may provide better insights into the mechanisms responsible for this pathogen's distribution in North American crayfish populations, and may improve predictions of further spread of various *A. astaci* genotypes. Understanding of such processes may facilitate efforts to limit the impact of these exotic species. Additional introductions of new *A. astaci* hosts should also be prevented, particularly through informing the general public to avoid releases of species available through ornamental pet trade (apparently the most important entry pathway for exotic crayfish in Europe at present; Chucholl, 2013; Peay, 2009). In the Netherlands, however, where numerous exotic crayfish species already live in a small area characterized by numerous interconnected water bodies, management of these aquatic invaders is difficult and presents a particular challenge for conservation and reintroduction of indigenous crayfish populations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2014.06.002.

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