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Status of *Pacifastacus leniusculus* and its role in recent crayfish plague outbreaks in France: improving distribution and crayfish plague infection patterns

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Abstract

Aphanomyces astaci, the crayfish plague pathogen, is responsible for mass mortalities in native European crayfish stocks. Its persistence and spread across Europe has been facilitated by the presence of invasive North American crayfish species, which act as asymptomatic vectors of this pathogen. In France, some recent mass mortalities have involved the pathogenic strain harboured by the invasive signal crayfish, *Pacifastacus leniusculus,* which may share habitats with the autochthonous white-clawed crayfish, *Austropotamobius pallipes*. To improve the efficiency of conservation management of *A. pallipes*, we have (i) updated information on the distribution of *P. leniusculus* populations in France based on data collected by the ONEMA (French National Agency for Water and Aquatic Environments), (ii) studied the distribution and prevalence of the crayfish plague pathogen within *P. leniusculus* populations throughout the country, and finally (iii) genotyped the strains responsible for several recent mass mortalities in *A. pallipes* populations. In total, 1658 populations of the signal crayfish were recorded in France; 1554 of these in streams and 104 in ponds. In 2014, this species was present in 80 of 95 French departments. Among the 1131 analyzed *P. leniusculus* individuals from 94 localities, 255 individuals (23%) tested positive for *A. astaci* presence. Infected individuals were detected in 63% of studied populations. Local prevalence varied highly among populations, ranging from 0% (no detection of *A. astaci*) up to 90% in the most infected ones. Out of five mass mortalities characterized in France in 2014–2015, four involved the strain from genotype group B, specific to *P. lenisuculus*. Our results confirm that the widespread signal crayfish serves as a key reservoir of *A. astaci* in France and therefore represents a serious danger for native crayfish species, especially the white-clawed crayfish.

Key words: signal crayfish, invasive species, oomycetes, mass mortality, *Aphanomyces astaci*

Introduction

The introduction of non-indigenous crayfish species (NICS) outside of their native ranges constitutes a major threat to freshwater biological diversity, with significant negative impacts on their native counterparts and at the ecosystem level (Souty-Grosset et al. 2006). European freshwater ecosystems have been particularly affected since the first documented introduction of the American crayfish *Orconectes limosus* to Pommerania (presently Poland) in 1890. To date, eight NICS of American origin have been successfully introduced into Europe via human activities (Kouba et al. 2014).

In France, the database on crayfish species distribution was developed by the French National Agency for Water and Aquatic Environments (ONEMA) in 1978. Each decade, crayfish distribution

is updated based on data collected at the departmental level by the ONEMA departmental service. In the last investigation, Collas et al. (2007) reported the presence of three American crayfish species in France: the spiny-cheek crayfish *O. limosus*, the signal crayfish *Pacifastacus leniusculus* and the red clawed crayfish *Procambarus clarkii*. Although transport of live non-native crayfish species is forbidden in France, two additional American crayfish species, *Orconectes juvenilis* and *Orconectes immunis*, are now found in French freshwater hydrographic systems (Kouba et al. 2014). Among these five species, *P. leniusculus* is considered the most likely species to affect native crayfish stocks in France. This crayfish species may inhabit the same headwater habitats as the endangered white-clawed crayfish *Austropotamobius pallipes* (Bramard et al. 2006), and may compete with the autochthonous species either directly, or impact them indirectly via the transmission of pathogens (Alderman and Polglase 1986, 1988; Weinländer and Füreder 2012; Chucholl 2016). *Pacifastacus leniusculus* was first introduced to Europe (Sweden) in 1959 in an attempt to replace declining *A. astacus* populations (Souty-Grosset et al. 2006). Now it is the most widespread NICS in Europe and is present in at least 29 territories (Kouba et al. 2014). In France, it was first introduced in 1974 in Thonon-les-Bains (Haute-Savoie) (Souty-Grosset et al. 2006) and by 2006 it was recorded from about 1000 sites in 73 out of 95 French departments (Collas et al. 2007). Because of the detrimental effects of signal crayfish introductions on native biota and its high potential invasiveness in most of Europe, it has been included in the list of invasive alien species (IAS) of Union concern (EU regulation No 1143/2014).

The three widespread NICS of North American origin in Europe (*O. limosus, P. clarkii* and *P. leniusculus*) are proven vectors of *A. astaci*, which is responsible for mass mortalities in European crayfish stocks (Alderman et al. 1990; Holdich et al. 2009; Kozubíková et al. 2009; Kozubíková-Balcarová et al. 2014; Rezinciuc et al. 2014). Recent evidence confirms that the less widespread NICS introduced more recently to European waters are also often carriers of this pathogen (Schrimpf et al. 2013; Keller et al. 2014; Tilmans et al. 2014). Due to its devastating impact, *A. astaci* has been included among the 100 worst invasive alien species (Lowe et al. 2000).

The use of molecular diagnostic methods for detection of the crayfish plague pathogen has confirmed *A. astaci* presence in French populations of *P. leniusculus*, *P. clarkii* and *O. immunis* (Filipová et al. 2013). However, their contribution to disease transmission likely varies substantially, depending on the distribution and pathogen prevalence patterns in different carrier species. To assess the transmission pathways more directly, it is possible to genotype the pathogen strains involved in mass mortalities, and link them to the most likely carrier species (Grandjean et al. 2014; Rezinciuc et al. 2014). Microsatellite markers, which allow direct identification of *A. astaci* genotype groups from infected individuals (Grandjean et al. 2014), are commonly used for this purpose (e.g., Vrålstad et al. 2014; Collas et al. 2016; Maguire et al. 2016; Kaldre et al. 2017; Mrugała et al. 2017). Among the five different *A. astaci* genotype groups (A–E) described so far from crayfish species (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011a), two have been isolated from *P. leniusculus* (groups B and C).

In the largest study screening *P. leniusculus* for *A. astaci* infection (with 513 individuals analyzed from 45 French populations), Filipová et al. (2013) reported the pathogen from 24 (53%) of the studied populations, with an infection prevalence within populations reaching up to 80%. This indicates a major threat of crayfish plague transmission from *P. leniusculus* to French crayfish populations, as is the case in other regions, e.g., Sweden (Bohman et al. 2006). Indeed, one of the earlier disease outbreaks analysed by microsatellite markers could have been linked to the *A. astaci* genotype group B associated with *P. leniusculus* (Grandjean et al. 2014), as was the recently reported major outbreak that decimated one of the largest *A. pallipes* populations in France (Collas et al. 2016). On the other hand, *A. astaci* genotype groups associated with *O. limosus* and *P. clarkii* cause crayfish mass mortalities in France as well (Grandjean et al. 2014). Assessing the relative contribution of different *A. astaci* genotypes to the outbreaks is thus important for understanding the dynamics of this disease.

The aims of this paper are: (i) to update the previous investigation on distribution of the signal crayfish in France (Collas et al. 2007), for the first time using geolocalisation of *P. leniusculus* populations; (ii) to complete the preliminary study performed by Filipová et al. (2013) on the *A. astaci* infection status of French signal crayfish populations; (iii) to reevaluate the risk of crayfish plague transmission imposed by this alien crayfish species; and finally (iv) to genotype *A. astaci* strains from a series of outbreaks that took place in 2014 and 2015 in France, and hence to quantify the contribution of strains transmitted from *P. leniusculus* to losses of indigenous crayfish populations. This study improves knowledge on spread and infection patterns for this species and thus contributes towards the conservation and management of endangered indigenous crayfish, in particular the white-clawed crayfish species *A. pallipes*.

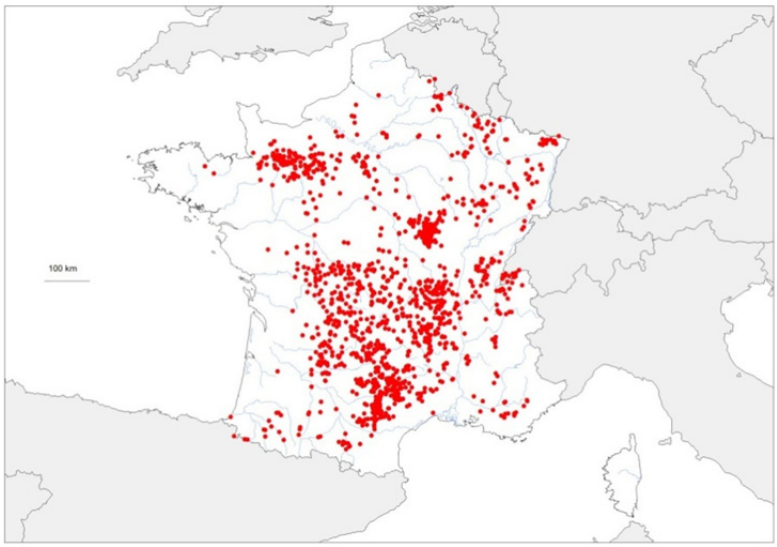

Figure 1. Distribution of the invasive signal crayfish *Pacifastacus leniusculus* populations in France in 2014 according to ONEMA inventories. Each population was characterised by geographic coordinates and is represented by a red dot.

Material and methods

Distribution of P. leniusculus in France

The last investigation of crayfish distribution in France was performed in 2006 (Collas et al. 2007) but only the presence or absence of crayfish species were reported at the departmental level. From 2006 to 2014, a new screening protocol for crayfish presence was performed by ONEMA. From early June to September each year, crayfish were caught at night using baited traps or by electrofishing and were determined to species. Indigenous crayfish species (ICS) were re-released, and NICS were removed from the system following French law on invasive species. For this new inventory, the quality of data has been improved by the geolocalisation of each crayfish population. A map of species distribution at the country level was then generated in ArcGis 10.0.

Screening for crayfish plague pathogen prevalence in P. leniusculus populations

In total, 1131 signal crayfish individuals sampled between 2008 and 2012 by the ONEMA departmental service from 94 localities in France (Supplementary material Table S1) were analysed for *A. astaci* infection; these sites cover a significant portion of the regions most invaded by this species (compare Figures 1 and 2). Preliminary results obtained by Filipová et al. (2013) on 513 individuals from 45 populations were included in this study. Most individuals were caught from running waters, especially brooks and several rivers. After sampling, crayfish were stored in 96% ethanol until further use.

Tissues from one half of the soft abdominal cuticle and one uropod were dissected from each crayfish using sterile tools, collected in a single 1.5 mL tube, dried and stored in a deep freezer at −80 °C. These samples were then tested for *A. astaci* infection following Filipová et al. (2013). We used DNeasy tissue kit (Qiagen) to isolate DNA from the cuticle samples immersed in 360 µL of Buffer ATL and crushed by stainless steel beads (1.6 mm diameter) a BBX24B Bullet Blender (Next Advance) for 10 min. DNA extractions otherwise followed the spin-column protocol of the DNeasy tissue kit but the volume of the reagents was doubled.

The presence of *A. astaci* genetic information in the DNA isolate was tested by the TaqMan MGB real-time PCR assay (Vrålstad et al. 2009) in 25 µL reaction volumes, using a LightCycler 480 Instrument (Roche). The relative level of infection by the pathogen was determined by the strength of the qPCR signal and recalculated to the corresponding amount of PCR-forming units (PFUs) in the reaction, then expressed as semi-quantitative agent levels (following Vrålstad et al. 2009; Kozubíková et al. 2011b).

We estimated the prevalence (with 95% confidence intervals) of *A. astaci* in our studied populations using the function "epi.conf" included in the library epiR (Stevenson et al. 2013) from the statistical package R v. 3.0 (R Core Team 2013). We also assessed spatial autocorrelation in the data by examining pairs of sample locations. By measuring the distance between two locations and plotting the squared difference between infestation values in the respective populations, we evaluated whether the pattern correspond to clustered, dispersed, or random distribution

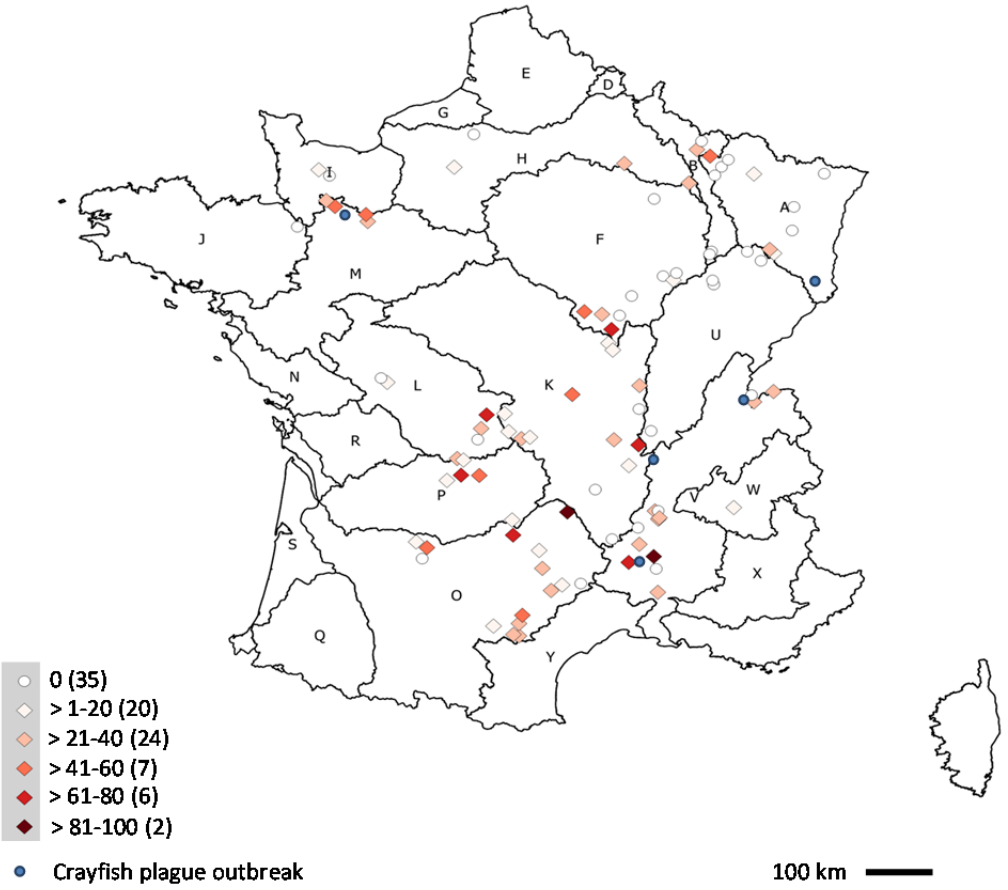

Figure 2. Map of hydrographic regions (in capital letters) showing the distribution of analysed populations for crayfish plague detection and the prevalence of infection in percentages (classified into six categories). Number of analysed populations for each prevalence category is indicated in brackets. Blue dots indicate crayfish plague outbreaks in native crayfish populations. A Rhine, B Meuse, D tributaries of Rhine, E Escaut and coastal streams at Bresle, F Seine, G Coastal streams from Artois Picardie to Seine, H Seine and Oise, I Coastal streams from Seine (excluded) to Loire Bretagne, J Bretagne, K Loire from its spring to Vienne (excluded), L Loire from Vienne to Maine (excluded), M Loire from Maine to the sea, N Coastal streams in south of Loire, O Garonne, P Dordogne, Q Adour, R Charente, S Coastal streams, U Saône, V Rhône, W Isèr, X Durance, Y Mediterranean streams.

(Devevey and Brisson 2012). We also calculated the value of the Moran's I, z-score and p-value associated with that index value. The analysis was performed in ArcGis 10.0

Genotyping of A. astaci strains in crayfish outbreaks

Between 2014 and 2015, five outbreaks of crayfish plague were documented in France (2014: La Lucelle, department Haut-Rhin; La Noullée, Sarthe; Le Curraize, Loire; Le Grozon, Corrèze; 2015: Le Buizin, Haute-Savoie) (Figure 2). Two individuals from each crayfish outbreak were genotyped with 9 microsatellite loci (Aast 2, 3, 6, 7, 9, 10, 12, 13 and 14) following protocols in Grandjean et al. (2014). The amplification was performed on an iCycler (Bio-Rad) for all tested microsatellite loci with the following conditions: 35 cycles of 95 °C for 30 s, 54 °C for 90 s, and 72 °C for 60 s, followed by a final extension of 5 min at 72 °C. Following the amplification, the samples were diluted by adding ultrapure Milli-Q water. To each sample of 0.5 µL of the diluted PCR products, the mix containing 0.5 µL of GENESCAN 500 ROX (Applied Biosystems) and 9 µL of deionized formamide was added. Microsatellite alleles were detected on an ABI 3130XL Genetic Analyser (Applied Biosystem) and scored using the GENEMAPPER version 3.7 software (Applied Biosystems).

The resulting multilocus genotypes (allele sized at individual loci) were compared by eye with the patterns obtained from reference strains representing all known genotype groups of *A. astaci* (Grandjean et al. 2014).

Results

P. leniusculus distribution in France

In total, 1658 signal crayfish populations were recorded in France (Figure 1), including both historical data and those obtained during the screening between 2006 and 2014. Of these populations, 1554 were in streams and 104 in ponds. By 2014, this species was present in 80 out of 95 French departments. *P. leniusculus* was recorded for the first time in the following departments: Bouches-du-Rhône (southeastern France), Calvados, Côtes d'Armor (northwestern France), Landes, Meurthe-et-Moselle (northeastern France), Tarn-et-Garonne (southeastern France), and Val d'Oise (north central France).

At the country level, signal crayfish are unevenly distributed (Figure 1). The species seems scarce or even completely absent across the Atlantic coast, from Finisterre in the north to Landes in the south. *P. leniusculus* was also underrepresented in the regions Nord and Picardie (northern France). Although populations of signal crayfish seem to be rather scattered in the eastern part of France, regions such as the north of the Lorraine and the western slope of the Vosges in Alsace harbor numerous *P. leniusculus* populations. The entire Massif Central, from the Plateau de Millevache (Limousin) to the Cévennes, is currently colonized by *P. leniusculus*. Its presence was recorded in all of its 22 departments and seems to be the most abundant at the national level (Table 1). *P. leniusculus* was also widespread in regions of Basse-Normandie (Manche, Orne) in the northwest, Morvan (Nièvre, Yonne) in the northern part of central France, and in several departments from the Rhône-Alpes region (Haute-Savoie, Ain) in the southeast of France.

Table 1. List of French departments with the highest number of *Pacifastacus leniusculus* populations arranged by habitats.

Department	Number of populations	Streams	Ponds
Allier	40	36	4
Aveyron	140	129	11
Cantal	75	75	–
Corrèze	37	35	2
Loire	75	73	2
Haute-Loire	65	57	8
Nievre	134	129	5
Orne	104	94	10
Puy de Dôme	74	72	2
Tarn	86	85	1

Aphanomyces astaci prevalence in P. leniusculus populations

Infection rate within populations

Out of 1131 signal crayfish analysed (Table S1), 255 individuals (23%) tested positive for *A. astaci* presence (agent level A2–A5). Infected individuals were detected in 59 (63%) of the 94 studied populations. Local prevalence varied highly among populations, ranging from 0% (no detection of *A. astaci* in analysed individuals) up to 90% (Landone population in Auvergne region) (Table S1). In the majority of signal crayfish individuals (876), the pathogen was either not detected (agent level A0; 744 individuals) or qPCR analyses indicated a presence of traces of *A. astaci* DNA not considered as a reliable positive detection of the pathogen (agent level A1; 132 individuals). 178 individuals with agent level A2 were recorded. Agent level A3 was found in 67 individuals, A4 and A5 in 5 individuals each.

The distribution of *P. leniusculus* populations with the highest crayfish plague prevalence (over 50%) was scattered (Figure 2). These were located in Auvergne, Limousin (Central France), Rhône-Alpes (Eastern France), Basse-Normandie (northwestern France), Midi-Pyrénées (Southern France) and the Lorraine region (Northeastern France) (Table S1, Figure 2). In some regions, *A. astaci*-positive individuals were found in the majority of analysed local populations, such as in Limousin (9 out of 10 populations infected), Rhône-Alpes (14/17), and Basse-Normandie (5/7). On the other hand, relatively low numbers of infected individuals were detected in most populations from the Lorraine region, where 6 out of 13 analysed populations were shown to be infected. Similarly, we detected *A. astaci* in only 2 out of 8 populations in Champagne-Ardenne (Table S1, Figure 2). In Languedoc-Rousillon, *A. astaci* was not detected; however, only 25 individuals from three populations were analysed from that region.

Geographical distances had no effect on the infestation rate in *P. leniusculus* populations. Moran's I coefficient, testing the correlation between the distance in kilometres and the prevalence, was not significantly different (lm = 0.8995, p = 0.35) from a random distribution.

Genotyping of *A. astaci* strains in crayfish outbreaks

Out of five crayfish mass mortalities recorded in 2014–2015, from which we genotyped the crayfish plague pathogen, microsatellite allele sizes matched the genotype of group B (strain originating from *P. leniusculus*) in four of them (La Lucelle, Haut-Rhin; La Noullée, Sarthe; Le Curraize, Loire; Le Grozon, Corrèze). The outbreak in Le Buizin from Haute-Savoie department was due to an *A. astaci* strain from the genotype group E, originally isolated from *O. limosus* (Kozubíková et al. 2011a).

Discussion

The range of *P. leniusculus* continues to expand in the French hydrographic system, with 7 departments newly colonized since 2006. In total, 1658 signal crayfish populations were recorded in France, a 60% increase since 2006 (Collas et al. 2007) due to its natural spread but also to illegal human translocation. The number of signal crayfish populations seems highest in the Massif Central region, from the Plateau de Millevache until the Cévennes, the regions where signal crayfish were massively introduced in the 1970s. Between 1974 and 1977, when any regulatory legislation was lacking, 18 000 juvenile signal crayfish of Swedish origin were introduced in several acclimatisation attempts in the Haute-Savoie, Ain and Yonne departments (Laurent 1983). This species was subsequently introduced on various occasions into both closed water bodies and open waters (Vigneux 1980). The regions Nord and Lorraine are also well-colonised by signal crayfish. Historical data has shown that introduction trials were performed during the 1970s in the Meuse department (Laurent 1983).

Overall, this species is rarely found in ponds, these localities accounting for only 6% of all recorded signal crayfish populations in France. Similar findings, i.e. signal crayfish being most prevalent in lotic habitats, were also reported from Austria and Germany (Weinländer and Füreder 2012; Chucholl 2016). Owing to a rapid spread of *P. leniusculus* and an increase in the number of established populations, the transportation of live specimens has been forbidden in France since 1983. Moreover, any introduction attempt is punishable by a fine of 9000 euros according to French legislation (Article L 432-10, code de l'environnement (2016)). In order to control signal crayfish populations, local fishing federations organise fishing days to promote the removal of this species from the colonised watercourses but this activity is not efficient (Peay 2009).

Our study detected *A. astaci* in 63% of analysed signal crayfish populations across the whole country; with prevalence rate fluctuating greatly among them from 0% to 90 %. This pattern, with some populations apparently little infected but others with very high prevalence, is consistent with the smaller dataset analysed by Filipová et al. (2013) from France, as well as with another recent study focusing on signal crayfish populations in the United Kingdom (James et al. 2017). *A. astaci* has been confirmed in signal crayfish populations in numerous other countries, including the Czech Republic and Slovakia (Kozubíková et al. 2011b), Germany (Chucholl and Schrimpf 2016), Hungary (Kozubíková et al. 2010), Spain (Diéguez-Uribeondo 2006), Sweden (Svoboda et al. 2014), Finland (Strand et al. 2012; Aydin et al. 2014), Norway (Vrålstad et al. 2011) and the Netherlands (Tilmans et al. 2014) but not in one analysed population in Denmark (Skov et al. 2011).

The prevalence of the disease in France is not correlated with the geographical distance among populations and differs greatly among and within regions. This is probably due to the very diverse origin of the introduced populations as well as founder effects following anthropogenic translocations (e.g., Grandjean and Souty-Grosset 1997; Diéguez-Uribeondo 2006; Kozubíková et al. 2009; Tilmans et al. 2014, Petrusek et al. 2017). Indeed, if only a few individuals from an intermediately infected population are introduced into a new environment, it is possible that those individuals are the uninfected ones.

Several other non-exclusive hypotheses could explain the fluctuation of prevalence of *A. astaci* observed among signal crayfish populations, such as the influence of water parameters on the development of the pathogen. Both growth and sporulation capacity of *A. astaci* are strain- and temperature-dependent (Diéguez-Uribeondo et al. 1995). Strains from genotype groups A and B prefer cold waters (4–21 °C) and are virulent at 10 °C whereas strains from genotype group D, isolated from a host species specific to warmer waters, have higher radial growth rates in temperatures ranging between 20–26 °C (Diéguez-Uribeondo et al. 1995).

The heterogeneity in prevalence found between populations could also be linked to crayfish population densities, as a dense population would favour pathogen spread. The regions corresponding to the primary introduction sites (Auvergne, Midi-Pyrénées, Rhône-Alpes, and Limousin) are densely populated by *P. leniusculus*, and have a high prevalence of *A. astaci*. However, pathogen prevalence may also vary over time (Matasová et al. 2011), so interpretation of patterns from a single sampling event must be made with caution.

Our results further strengthen the evidence that the signal crayfish is the most important reservoir for the crayfish plague pathogen in France. An *A. astaci* genotype group originating from this host species was responsible for four out of five disease outbreaks, confirming the high virulence of this genotype group (Diéguez-Uribeondo 2006; Makkonen et al. 2012, 2014; Becking et al. 2015). Therefore, the signal crayfish represents a serious threat to native crayfish populations, especially the white-clawed crayfish *A. pallipes.* Indeed, this endangered species occupies a very similar ecological niche to the signal crayfish (Kopp et al. 2010; Weinländer and Füreder 2012; Chucholl 2016).

Our findings highlight sites requiring special attention, where populations of signal crayfish are highly infected, and in particular those in close vicinity to white-clawed crayfish populations (see also James et al. 2017). As it is practically impossible to rid ecosystems (especially lotic ones) of signal crayfish, the risk of crayfish plague transmission can be very high in these highly-infected zones. Native crayfish populations in these high-risk zones could be translocated to safer areas, i.e., "ark sites" (Peay 2009). If needed, the eradication, or at least control, of the most infected non-native crayfish populations could also be considered (Oidtmann et al. 2006; Peay and Dunn 2014). We hope that our results will contribute to a better awareness on the potential impact of infected alien crayfish populations and to improving the efficiency of conservation management of the indigenous white-clawed crayfish.

As crayfish plague spores may be accidentally transmitted through water or contaminated equipment (boots, wet nets, etc.) during fishery management and other activities (see also review in Svoboda et al. 2017; Jussila et al. 2014), it is essential to implement a routine disinfection protocol of used materials. Fish aquaculture practices located at the basin heads should favour the precautionary principle in order to avoid transmission of the spores through fish species that could act as potential vectors. Systematic control of *A. astaci* presence (i.e. by analysing water with eDNA method; Strand et al. 2011, 2012) could be set up at the sources prior to restocking, in order to determine the risk of dissemination with contaminated water or fish themselves (Oidtmann et al. 2002).

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Supplementary material

The following supplementary material is available for this article:

Table S1. Number of analysed and infected individuals, prevalence of the crayfish plague pathogen *Aphanomyces astaci* and agent level in infected individuals are given for each population.

This material is available as part of online article from:
http://www.aquaticinvasions.net/2018/Supplements/AI_2018_Grandjean_etal_Table_S1.xlsx