

Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe

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Abstract

Crayfish of North American origin are amongst the most prominent high-impact invasive invertebrates in European freshwaters. They contribute to the decline of European native crayfish species by spreading the pathogen causing crayfish plague, the oomycete *Aphanomyces astaci*. In this study we validated the specificity of four quantitative PCR (qPCR) assays, either published or newly developed, usable for environmental DNA (eDNA) screening for widely distributed native and non-native crayfish present in Central Europe: *Astacus astacus*, *Pacifastacus leniusculus*, *Faxonius limosus* and *Procambarus virginalis*. We then conducted an eDNA monitoring survey of these crayfish as well as the crayfish plague pathogen in a wide variety of habitat types representative for Central and Western Europe. The specificity of qPCR assays was validated against an extensive collection of crayfish DNA isolates, containing most crayfish species documented from European waters. The three assays developed in this study were sufficiently species-specific, but the published assay for *F. limosus* displayed a weak cross-reaction with multiple other crayfish species of the family Cambaridae. In the field study, we infrequently detected eDNA of *A. astaci* together with the three non-native crayfish species under examination. We never detected eDNA from *A. astaci* together with native crayfish, but in a few locations eDNA from both native and non-native crayfish was captured, due either to passive transport of eDNA from upstream populations or co-existence

in the absence of infected crayfish carriers of *A. astaci*. In the study, we evaluated a robust, easy-to-use and low-cost version of the eDNA sampling equipment, based mostly on items readily available in garden stores and hobby markets, for filtering relatively large (~5 l) water samples. It performed just as well as the far more expensive equipment industrially designed for eDNA water sampling, thus opening the possibility of collecting suitable eDNA samples to a wide range of stakeholders. Overall, our study confirms that eDNA-based screening for crayfish and their associated pathogen is a feasible alternative to traditional monitoring.

Keywords

crayfish plague, eDNA monitoring, eDNA sampling methods, quantitative PCR, TaqMan assay validation

Introduction

Environmental DNA (hereafter eDNA) is commonly defined as genetic material obtained directly from environmental samples (soil, sediment, water) without any obvious signs of the biological source material (Thomsen and Willerslev 2015). In water samples, eDNA typically originates from single-celled uncultured microorganisms or, in the case of multicellular taxa, from shed cells, faeces, mucus, body fluids, gametes, spores or other propagules (Strand et al. 2014; Deiner et al. 2016; Mächler et al. 2016) or even from recently dead and decomposing organisms (Strand et al. 2019).

During the past decade, different concepts of eDNA analyses have become established for various purposes such as monitoring endangered and elusive targets, invasive species, as well as parasites and pathogens (Kirshtein et al. 2007; Thomsen et al. 2012a; Takahara et al. 2013; Rusch et al. 2018; Strand et al. 2019). There are two essentially different approaches to eDNA monitoring: either broad spectrum metabarcoding for bio-assessments of whole communities (Thomsen et al. 2012a; Valentini et al. 2016; Ruppert et al. 2019) or more targeted approaches for the detection and quantification of one or several species of interest (Jerde et al. 2011; Thomsen et al. 2012b), usually using species-specific quantitative real-time PCR (qPCR) or droplet digital PCR (ddPCR). Since eDNA has a relatively short half-life in the water column of aquatic systems (Dejean et al. 2011), positive detection suggests that the targeted organism is either present or has been present within the system very recently.

One of the pathogens for which monitoring methods based on eDNA have been developed is the oomycete *Aphanomyces astaci* Schikora, the causative agent of crayfish plague (Strand et al. 2011, 2012, 2014; Robinson et al. 2018; Wittwer et al. 2018). Since its initial introduction into Europe in the late 1850s (Alderman 1996), and reinforced by subsequent introductions of several Non-Indigenous Crayfish Species (NICS) of North American origin (Holdich et al. 2009), crayfish plague has ravaged the continent and led to mass mortalities of native crayfish (Alderman 1996; Holdich et al. 2009). *Aphanomyces astaci* is usually carried as a benign infection by its natural crayfish hosts from North America, where both originate. However, crayfish indig-

enous to Europe usually lack efficient defence mechanisms to resist this pathogen and thus whole populations tend to be eliminated as a result of crayfish plague outbreaks (Söderhäll and Cerenius 1999; Holdich et al. 2009; Vrålstad et al. 2014). This explains why *A. astaci* is a disease listed by the World Organisation for Animal Health (OIE 2019) and featured on the list of the “world’s 100 worst invasive species” (Lowe et al. 2004).

American crayfish species, such as the spiny cheek crayfish *Faxonius limosus* (Rafinesque, 1817), the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) and the red swamp crayfish *Procambarus clarkii* (Girard, 1852), were originally introduced into Europe for stocking or aquaculture purposes (Holdich et al. 2009). Others, such as the marbled crayfish *Procambarus virginalis* Lyko, 2017, reached European waters through the pet trade (Chucholl 2013; Kouba et al. 2014). All species listed above pose a threat to native European crayfish species and are therefore subject to the EU Regulation on the prevention and management of the introduction and spread of invasive alien species (Regulation (EU) No 1143/2014).

The marbled crayfish, *P. virginalis*, is causing great concern outside of Europe, too. This triploid species seems to have emerged as a thelytokous parthenogenetic form of *Procambarus fallax* (Hagen, 1870), possibly from the pet trade (Gutkunst et al. 2018; Martin et al. 2010). Thus, it produces female-only offspring and a single individual is required to establish a new population. It has been shown to thrive in a very broad range of habitats, recently demonstrated in Madagascar (Andriantsoa et al. 2019).

When non-indigenous crayfish are present, the only conceivable option to eradicate crayfish plague is by treating the entire waterbody with pesticides such as Betamax-VET (Sandodden and Johnsen 2010). This procedure kills the crayfish hosts and subsequently also the crayfish plague pathogen which depends on its host for long-term survival (Söderhäll and Cerenius 1999). However, this is only applicable to smaller aquatic habitats (Peay et al. 2019) and, even there, it is an extremely costly and devastating undertaking, often not compliant with local legislation. Therefore, mitigation strategies must be employed to preserve and protect Indigenous Crayfish Species (ICS) and their natural environment. These mitigation strategies can include the prohibition of fishing in certain areas or the enforcement of decontamination protocols for fishing gear. They could also encompass the creation and management of the so-called “ark sites”, where introduction of neither the alien crayfish nor the disease is likely (Peay 2009a). When creating such ark sites or planning restocking and rescue transfers, precise knowledge about the distribution of crayfish plague vectors and presence or absence of the crayfish plague agent in the vicinity is required. For this purpose, the eDNA methodology is a particularly suitable tool (Cowart et al. 2018; Strand et al. 2019).

Recent research has focused on developing eDNA monitoring for early alert of NICS and *A. astaci*, as well as for efficient biomonitoring of ICS. The main goals are safeguarding indigenous crayfish while limiting the spread of both NICS and crayfish plague pathogen (Strand et al. 2014, 2019; Agersnap et al. 2017; Cai et al. 2017; Vrålstad et al. 2017; Harper et al. 2018; Wittwer et al. 2019).

In this study we demonstrate the applicability of eDNA-based screening for crayfish and the crayfish plague pathogen in a wide range of aquatic habitats in Czechia, a Central European country with a long tradition of crayfish conservation and research. Three European crayfish species, the noble crayfish *Astacus astacus* (Linnaeus, 1758), the stone crayfish *Austropotamobius torrentium* (Schrank, 1803) and the narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) are found in local waters. The two former species are native to the country, the latter being introduced from Eastern Europe to multiple localities in the late 19th century (Štambergová et al. 2009). Crayfish plague has caused large-scale mortalities of native crayfish in the area since the 1890s (Kozubíková et al. 2006). Although not considered a conservation problem throughout most of the 20th century, crayfish plague outbreaks, caused by *A. astaci* genotypes associated with different North American host taxa (Grandjean et al. 2014), are at present rampant in the country (Kozubíková et al. 2008; Kozubíková-Balcarová et al. 2014; Mojžišová et al. 2020).

Czech waters host three documented North American crayfish species. *Faxonius limosus* that invaded the Elbe river as far back as the 1960s (Petrusek et al. 2006) and *P. leniusculus*, introduced for fishery purposes in 1980 (Filipová et al. 2006), are both widespread in at least some regions of the country (Kouba et al. 2014; Mojžišová et al. 2020). *Procambarus virginalis* has recently been documented from two sites, most likely resulting from aquarium releases (Patoka et al. 2016), but there is a high probability that other established populations of *P. virginalis* are yet waiting to be discovered. All these species are confirmed carriers of *A. astaci* (Svoboda et al. 2017). Infections of Czech populations have been documented for *P. leniusculus* and *F. limosus* (Kozubíková et al. 2009), but not for *P. virginalis* (Patoka et al. 2016).

Native and non-native crayfish populations can be found in a wide range of diverse habitats in Czechia: large and smaller rivers and streams as well as artificial still waters including fishponds, flooded quarries and reservoir lakes. There is a wealth of documented data on existing crayfish populations in lentic and lotic waterbodies in the country (Štambergová et al. 2009; Svobodová et al. 2012), together with data on the infection status by *A. astaci* in NICS populations (Kozubíková et al. 2009, 2011). Thus, Czechia is a suitable region to conduct a study focusing on eDNA-based detection of multiple NICS and their pathogen across a broad range of habitats.

The goal of the study presented here is two-fold: firstly, to validate the specificity of presumably species-specific qPCR assays for selected native and non-native crayfish present in Central Europe (Fig. 1). Three assays newly developed for this study and one previously published assay were tested against a broad panel of DNA isolates from various crayfish species present in Europe or available via the ornamental pet trade. Secondly, the presence of the crayfish plague agent *A. astaci* as well as its various crayfish hosts by means of eDNA analysis of water samples was evaluated. These were collected from various Czech localities and some from urban waters from Berlin (Germany) and Budapest (Hungary), which are representative for crayfish habitats in Central and Eastern Europe.



Figure 1. Crayfish species searched for by means of eDNA. Species clockwise from top left: *Faxonius limosus*, *Astacus astacus*, *Pacifastacus leniusculus*, *Procambarus virginalis*. Photos taken by A. Petrusek (Fl) and J. Rusch (Aa, Pl, Pv).

Methods

Study sites and populations

A full range of all relevant habitats for Central and Western Europe was covered, including large rivers and small streams, a thermal stream, natural lakes and man-made reservoirs, flooded quarries and fishponds (in total 32 localities; Suppl. material 1: Table S1). The majority of the samples (28) were taken in August 2017 at various waterbodies within Czechia, for which previous presence of crayfish was reliably known or presumed. The sampling sites were chosen to ensure that each one could be considered negative for at least some of the four target crayfish species, i.e. *F. limosus*, *P. virginalis*, *P. leniusculus* and *A. astacus*. None of the sites was within the known distribution area for stone crayfish in the country (Vlach et al. 2009; Petrusek et al. 2017a). Two samples were collected in December 2018 at two lakes in Berlin with a recently reported or assumed presence of both *P. virginalis* and *F. limosus* (Linzmaier et al. 2018; A. Mrugała, pers. comm.). Two additional water samples were obtained in January 2019 from a

stream in Budapest and its thermal tributary with a confirmed co-existence of the same two (and also additional) NICS (Szendőfi et al. 2018; A. Kouba, pers. comm.). Control eDNA samples were collected from an aquarium housing numerous marbled crayfish individuals, held at the Czech University of Life Sciences, Prague.

For comparison with eDNA results, crayfish were actively searched for at most sampling locations by manual examination of suitable shelters to confirm their *in-situ* presence. At the Czech sites containing NICS, we also attempted to obtain individuals to test for infection with *A. astaci*. After collection of samples for eDNA analysis, these crayfish were either captured directly at the sampling site on the same date or obtained from a nearby site within the same watercourse. Occasionally, we benefited from availability of such samples from previous recent fieldwork, assuming that the infection status of the NICS population does not change dramatically in a short time (Matasová et al. 2011). Crayfish plague diagnostics were carried out according to the method described in Vrålstad et al. (2009) with minor modifications (Mrugała et al. 2015). In brief: the soft abdominal cuticle and part of the tail fan of each crayfish were dissected and ground in liquid nitrogen. Total genomic DNA was then extracted using the DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany) and the DNA extracts were then screened using the qPCR protocol for detection of *A. astaci* described below.

eDNA sample collection and extraction

Water samples at Czech locations 1 to 28 were obtained according to Strand et al. (2019) by filtering up to 5 l of water through glass fibre filters (47 mm AP25 Millipore, 2 µm pore size; Millipore, Billerica, USA), utilising a portable peristaltic pump (Masterflex E/S portable sampler; Masterflex, Cole-Parmer, Vernon Hills, USA), tygon tubing (Masterflex) and an in-line filter holder (Millipore). The front end of the tube was fastened to the inside of a plastic box which was weighted with lead on the bottom. This box was lowered into the water between 2 m and 5 m from the water's edge or to the centre of smaller streams. Before the filter was placed into the holder, water was pumped through the tubes for several minutes to remove any sediments that could have been disturbed from the waterbed and thus prevent clogging of the filter (Strand et al. 2019). For sampling sites where less than 5 l of water was filtered due to filter clogging, the final volume is noted in Table 2. At each location, two filter samples were taken.

For the samples obtained at locations 29 to 32 (Berlin and Budapest) the same filters (47 mm AP25 Millipore, 2 µm pore size) were used. However, the filters were placed into filter cups (Nalgene Analytical Test Filter Funnel, 145-0045; Thermo Fisher Scientific, Waltham, USA) after removal of the original filter provided by the manufacturer. Pumping was carried out by attaching the provided filter-cup adapter to a ¾ inch garden water hose and a drill-operated pump (product code 1490-20; Gardena, Ulm, Germany) (Fig. 2). As opposed to the protocol described above, the filters and filter cups were submerged into the water since they were situated at the front end of the pumping system (Fig. 2). The samples from the aquarium with *P. virginalis* and from the Barát stream in Budapest (sites 31, 32) were obtained after transporting water



Figure 2. Drill-powered sampling equipment. The low-cost sampling equipment used in this study consisting of a drill-powered pump, single use forceps, filter cups and glass fibre filters. The pump depicted in the bottom right corner is one of many alternative models to the one used in this study.

from the location in disinfected 5 l containers. This water was stored in the dark at low temperatures but not frozen and was filtered upon arrival in the laboratory using the drill-operated pumping system described above.

Filters from locations 1 to 28 were submerged in 4 ml of cetyl trimethyl ammonium bromide (CTAB) buffer in individual 15 ml Falcon tubes immediately after filtration and subsequently stored on ice until their arrival at the laboratory where they were stored at -20°C prior to further analysis. Filters from locations 29 to 32 were placed into separate zip-lock bags containing ca. 70 g of silica gel following Carim et al. (2016), which ensured efficient desiccation, and stored in an opaque container until further analysis in the laboratory.

To prevent contamination of filters and accidental spreading of crayfish plague, a strict disinfection protocol was followed at each location. After filtering, all the equipment was submerged in, and filled with, a 10% chlorine bleach solution for a minimum of 15 minutes to break down any vital pathogen spores and residual eDNA. Then the tubes and filter holders were rinsed with a 5% sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) solution to neutralise the chlorine solution. Prior to water sample filtration, the equipment was thoroughly rinsed with ambient water from the sampling site. While using the drill-operated pumping system, separate tubing and filter holders were used at each respective sampling site, thus eliminating the concern for carryover contamination.

DNA isolation from the filters was performed according to the CTAB method described in Strand et al. (2019). In brief: the samples were lysed on CTAB buffer and proteinase K at 65°C for one hour, cleaned and separated with chloroform and then precipitated in isopropanol. The pellets were then re-suspended in TE-buffer.

Due to the large volume of eluate from each filter, the samples were split up into two subsamples (technical replicates) to bypass the volume restrictions caused by centrifuge size. These subsamples were subsequently processed separately. Each extraction process incorporated an environmental blank control and an extraction blank control as a precautionary measure to detect any potential contamination during the extraction (Strand et al. 2019).

Molecular detection of target species with qPCR

Molecular eDNA detection of all five target-species (the crayfish plague pathogen *A. astaci* and the crayfish *A. astacus*, *P. leniusculus*, *F. limosus* and *P. virginalis*) was based on TaqMan MGB qPCR assays, either published in the case of *A. astaci* (Vrålstad et al. 2009) and *F. limosus* (Mauvisseau et al. 2018) or developed in this study (*A. astacus*, *P. leniusculus* and *P. virginalis*).

Due to the absence of any published assay for *P. virginalis* while this study was being carried out, we designed a qPCR assay with species-specific primers and a minor groove binder (MGB) probe targeting the mitochondrial gene for the cytochrome c oxidase subunit I (COI) of this asexually reproducing, genetically uniform species (cf. GenBank reference sequence: JF438007). We have since learnt of the existence of a newly-published assay (Mauvisseau et al. 2019) which targets a very similar fragment of the COI gene and thus differs only marginally from the one developed by us.

High specificity of the primers–probe combination was first ensured by checking the variation of the potential primer and probe sites against COI sequences of all crayfish known to occur in European waters, both native and invasive, and various related crayfish species of the family Cambaridae, particularly those available from the pet trade (taxa listed in Suppl. material 2: Table S2). This was accomplished using Geneious version 11.0.1 (Biomatters Limited, Auckland, New Zealand) and MEGA 7.0.26 (Kumar et al. 2016) through visual comparison. The efficacy of the primers and probe was evaluated using the Primer Express software (Version 3.0.1, Applied Biosystems, Foster City, USA).

New assays, differing from those published in Agersnap et al. (2017), Dunn et al. (2017), Larson et al. (2017), Harper et al. (2018), Mauvisseau et al. (2018) and Robinson et al. (2018), were designed for *A. astacus* and *P. leniusculus*. These two assays were developed with particular regard to functionality on both the qPCR and the droplet digital PCR (ddPCR) platform (D.A. Strand, unpublished). However, in this study we have only tested the efficiency and efficacy of the assays on the qPCR platform. Sequences from individual crayfish from several European regions (including North American individuals for *P. leniusculus*; Petrusek et al. 2017b) obtained from GenBank were used to design the assays for *A. astacus* and *P. leniusculus*.

For *in-vitro* validation, to determine the specificity of the assays, we re-used a total of 29 DNA isolates from tissues of crayfish species from previous studies on diversity of both indigenous and non-indigenous crayfish species in Europe that involved COI

Table 1. Primers and probes used in the present study. The probes used are TaqMan MGB probes with either FAM or VIC reporter dyes.

Target species	Target marker	Primer/probe	Sequence (5'-3')	Reference
<i>Aphanomyces astaci</i>	ITS	forward	AAGGCTTGTGCTGGGATGTT	Vrålstad et al. (2009)
		reverse	CTTCTTGCGAAACCTTCTGCTA	Vrålstad et al. (2009)
		probe	FAM-TTCGGGACGACCC-MGBNFQ	Vrålstad et al. (2009)
<i>Astacus astacus</i>	COI	forward	CCCCTTTRGCATCAGCTATTG	current study
		reverse	CGAAGATACACCTGCCAAGTGT	current study
		probe	FAM-CTCATGCAGGCGCAT-MGBNFQ	current study
<i>Pacifastacus leniusculus</i>	COI	forward	GAGTGGGTACTGGATGAAGT	current study
		reverse	GAAGAAACACCCGCTAAATGAAG	current study
		probe	VIC-CAGCGGCTATTGCT-MGBNFQ	current study
<i>Faxonius limosus</i>	COI	forward	CCTCCTCTCGTTCTGCAAT	Mauvisseau et al. (2018)
		reverse	AACCCCTGCTAAATGCAACG	Mauvisseau et al. (2018)
		probe	FAM-CTCATGCAGGGGCATCAGTGG-MGBNFQ	Mauvisseau et al. (2018)
<i>Procambarus virginalis</i>	COI	forward	ACGGGCAGCTGGTATAACTATG	current study
		reverse	TCTCCTCCACCAGCAGGATC	current study
		probe	FAM-CCGCTATTGTGTTGGTCAGTA-MGBNFQ	current study

sequencing (Filipová et al. 2011; Chucholl et al. 2015; Petrusek et al. 2017a). We also used isolates from surveys of *A. astaci* infections in various carrier species (Tilmans et al. 2014; Mrugała et al. 2015) and crayfish plague outbreaks (Kozubíková-Balcarová et al. 2014) (see additional material, Suppl. material 2: Table S2). The identity of non-indigenous species was confirmed and variation at the target marker (COI) in most of these particular isolates was assessed by DNA barcoding in previous studies (Filipová et al. 2011; Mrugała et al. 2015). The isolate collection, used to test the assay specificity, contained most of the native crayfish known from Western, Central and Northern European countries and the Balkans (see distribution maps in Kouba et al. 2014), with the exception of narrowly-endemic lineages related to *A. torrentium* (Klobučar et al. 2013; Pârvulescu 2019) and the thick-clawed crayfish *Pontastacus pachypus* (Rathke, 1837).

Both newly-developed assays for *A. astacus* and *P. leniusculus*, as well as the published assay for *F. limosus* (Mauvisseau et al. 2018), were subjected to the same *in-vitro* validation procedure as the assay for *P. virginalis*, described above. To ensure optimal performance of all qPCR assays targeting crayfish, we determined the most suitable annealing temperatures through a temperature gradient from 56 °C to 63 °C and multiple primer-probe concentrations were evaluated. Our two objectives were to define the conditions when the assays show efficient amplification of the target DNA but minimal cross-reaction with DNA of related taxa and, if possible, to establish a common protocol for routine application of all assays.

The final protocol used for eDNA screening was identical for the detection of all four crayfish species. The undiluted and diluted samples were run in the following 25 µl reaction: 12.5 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, USA), 1.25 µl of each 10 µM primer (forward and reverse), 1.25 µl of 5 µM TaqMan MGB probe, 3.75 µl of PCR-grade water and 5 µl of DNA sample. The following qPCR cycling conditions were used: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 1 min.

For all species-specific crayfish assays, we followed recommendations for defining the limit of detection (LOD) and the limit of quantification (LOQ) in qPCR assays used for diagnostic analyses of genetically-modified organisms and microbiological pathogens in foodstuff, tissues and environmental samples (Berdal et al. 2008). These have also been used for previously-published assays for crayfish plague (Vrålstad et al. 2009) and freshwater crayfish (“the Norwegian approach” in Agersnap et al. 2017). Genomic DNA from all target species was extracted according to the protocol in Agersnap et al. (2017) and stock solutions of 50 ng.µl⁻¹ genomic DNA (measured using Qubit fluorometer; Invitrogen, Carlsbad, USA) from each species were used to prepare a four-fold dilution series of 13 standard dilutions. In an initial qPCR test, ≥ 3 replicates of the standard dilution 1–8 were run on a Stratagene Mx3005P with qPCR-conditions as described above, while the standard dilutions 9–13 were run in 20 replicates. A template concentration of approximately 1 DNA copy per PCR volume will yield a positive:negative ratio of 7:3 (70% detection success; Berdal et al. 2008). Thus, the copy number in the standard dilutions closest to 70% detection rate were then calculated with most probable number (MPN) calculations (Berdal et al. 2008) and the obtained copy number was then used to calculate copy numbers in the more concentrated standards. The LOD was established for each assay following the criteria that LOD is the lowest concentration that yields a probability of false negatives < 5% (Berdal et al. 2008; Vrålstad et al. 2009). The LOQ was established using the same acceptance level as set for qPCR quantification of the crayfish plague pathogen *A. astaci* (Vrålstad et al. 2009), with observed standard deviation < 0.5 for the Ct-values.

In order to detect *A. astaci* in both eDNA samples and crayfish tissues, we used the assay developed by Vrålstad et al. (2009) with modifications according to Strand (2013). Each undiluted and diluted sample was run in the following 25 µl reaction: 12.5 µl of TaqMan Environmental Master Mix 2.0, 2.5 µl of each 5 µM primer (forward and reverse), 1 µl of 5 µM TaqMan MGB probe, 1.5 µl of PCR-grade water and 5 µl of DNA sample. The following qPCR cycling conditions were used: an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 15 s and annealing at 62 °C for 30 s.

All qPCR analyses of the eDNA samples were carried out on an Mx3005P qPCR thermocycler (Stratagene, San Diego, USA) at the Norwegian Veterinary Institute, Oslo. The validation of crayfish assays concerning specificity tests against other crayfish species was performed on a BioRad iQ5 (Bio-Rad, Hercules, USA) thermocycler at the Faculty of Science, Charles University, Prague. An analysis of a subset of eDNA

isolates on the BioRad iQ5 thermocycler suggested comparable performance to that on Mx3005P.

As described above, each filter was divided into two technical replicates/subsamples. Both subsamples were analysed as 2x undiluted and 2x 10-fold diluted replicates, in total 4 qPCR replicates per filter. Results for each respective filter were considered positive, only if more than one of the four reactions yielded positive results. A cut-off value was set at Ct 41 following previous recommendations (Agersnap et al. 2017; Kozubíková et al. 2011; Strand et al. 2019) which means that any amplification occurring at or above this value was not considered a positive detection.

The presence or absence of qPCR inhibition was controlled by calculating the difference in Ct values (Δ Ct) between the undiluted and corresponding 10-fold diluted DNA replicates as described in Kozubíková et al. (2011) and Agersnap et al. (2017). In case of apparent inhibition (if Δ Ct < 2.82) the estimated eDNA copy number was based on the 10-fold diluted DNA replicates alone, while if Δ Ct > 3.82 (i.e. 10-fold dilution out of range), the estimation of eDNA copy number was based solely on the undiluted DNA replicates (see Suppl. material 4: Table S4 for observed inhibition). If none or only one of the relevant replicates were detected above LOQ, further quantification was not performed and thus qPCR inhibition was not possible to evaluate either.

Results

Optimising and validating the crayfish qPCR assays

We successfully developed new assays for *A. astacus*, *P. leniusculus* and *P. virginalis*. All three assays were apparently species-specific *in-silico* and, for the first two, we also confirmed this *in-vitro*. The assay for *P. virginalis* displayed weak cross-amplification of three other cambarid species (see below). While *in-silico* testing the assays and comparing sequences of the respective crayfish to their closest relatives, we observed the assay for *F. limosus* to differ from a closely-related species *Faxonius* cf. *virilis* (a lineage of the *F. virilis* complex known from Europe; Filipová et al. 2010) by only one mismatch in the forward primer and two mismatches in the probe and the reverse primer, respectively. For subsequent qPCR testing with a temperature gradient, we included DNA isolated from European *F. cf. virilis* (labelled *F. virilis* below). While using the PCR conditions (annealing temperature 56 °C) suggested by the authors (Mauvisseau et al. 2018), *F. limosus* and *F. virilis* DNA were amplified at Ct 17.92 and 24.62 respectively. An increase in annealing temperature to 60.5 °C resulted in amplification of *F. limosus* and *F. virilis* DNA at Ct 18.58 and 34.12 respectively, thus increasing the specificity of the assay, although still cross-reacting with *F. virilis*.

Ensuing specificity testing against the collection of all DNA isolates (Suppl. material 2: Table S2) was carried out at 60 °C. The assay for *F. limosus*, which amplified the DNA of the target taxon at Ct 17.7 to 18.5, also amplified DNA of isolates of the following species (lowest Ct stated): *F. virilis* (Ct 30.14), *F. margorectus* (Ct 36.32),

F. rusticus (36.74), *F. harrisonii* (Ct 40.72), *F. punctimanus* (Ct 40.86), *P. virginalis* (Ct 36.13), *P. zonangulus* (Ct 37.91) and *P. acutus* (Ct 35.79). The assay for *P. virginalis*, which amplified the DNA of the target taxon at Ct 18.3 to 23.33 (depending on the starting DNA concentration of isolate), also weakly cross-amplified DNA of isolates from *P. acutus* (Ct 37.29), *P. alleni* (Ct 38.22) and *P. clarkii* (Ct 39.41).

For all crayfish assays, LOD was experimentally established as 5 copies/PCR reaction with good margin; the observed detection success for 20 replicates of a standard dilution corresponding to ~2–4 copies per PCR reaction was between 90–100% (for details see Suppl. material 3: Table S3). Further, LOQ was established as 10 copies per PCR reaction, where the assays demonstrated acceptable repeatability with observed standard deviation for the Ct-values (Suppl. material 3: Table S3).

Environmental DNA monitoring

We detected eDNA of all surveyed crayfish species during our sampling effort (Fig. 3). We also detected eDNA of the crayfish plague pathogen *A. astaci* together with the three investigated non-native crayfish species, but only infrequently. More commonly, eDNA from non-native crayfish was detected alone (Fig. 3, Table 2). A full overview of the qPCR results and eDNA copy estimations is supplied in Suppl. material 4: Table S4.

From the total of 32 surveyed locations, eDNA from native *A. astacus* was unambiguously detected in seven (~22 %) locations. In two of these, however, a positive amplification only occurred in one out of two filter samples. At four locations, the eDNA results were corroborated by observation of *A. astacus* at the sampling sites (Table 2). Simultaneous detection of *A. astacus* and *F. limosus* eDNA was observed in two locations (7 – Všechný reservoir and 10 – Pšovka), eDNA from *A. astacus* and *P. leniusculus* was simultaneously detected in location 16 (Oslava). Environmental DNA from the crayfish plague pathogen *A. astaci* was never detected in samples that contained *A. astacus* eDNA. However, in location 10 (Pšovka), we caught specimens of *F. limosus*, whose tissue analyses showed low *A. astaci* prevalence (20%) and very low infection load (agent level 2, A2; Vrålstad et al. 2009).

Non-native *P. leniusculus* was detected by eDNA in eight locations (25%), all where the species was expected according to our prior knowledge (Suppl. material 1: Table S1). All detections occurred in both samples taken at the respective sampling sites. The eDNA results were corroborated by observation of signal crayfish at seven locations on the date of sampling. Environmental DNA from the crayfish plague pathogen *A. astaci* was detected in only two of the locations where *P. leniusculus* was detected (13 – Malše and 15 – Dračice). In these two locations, data from tissue analyses confirmed high prevalence (80% and 100%) and low to high infection load (up to A3 and A5, respectively). For three other *P. leniusculus* positive locations (16 – Oslava, 20 – Žďárka, and 22 – Staviště), the apparent absence of *A. astaci* eDNA was corroborated by no detection of the pathogen in screened crayfish individuals (Table 2). Generally, *P. leniusculus* was the only crayfish species detected through eDNA at the respective

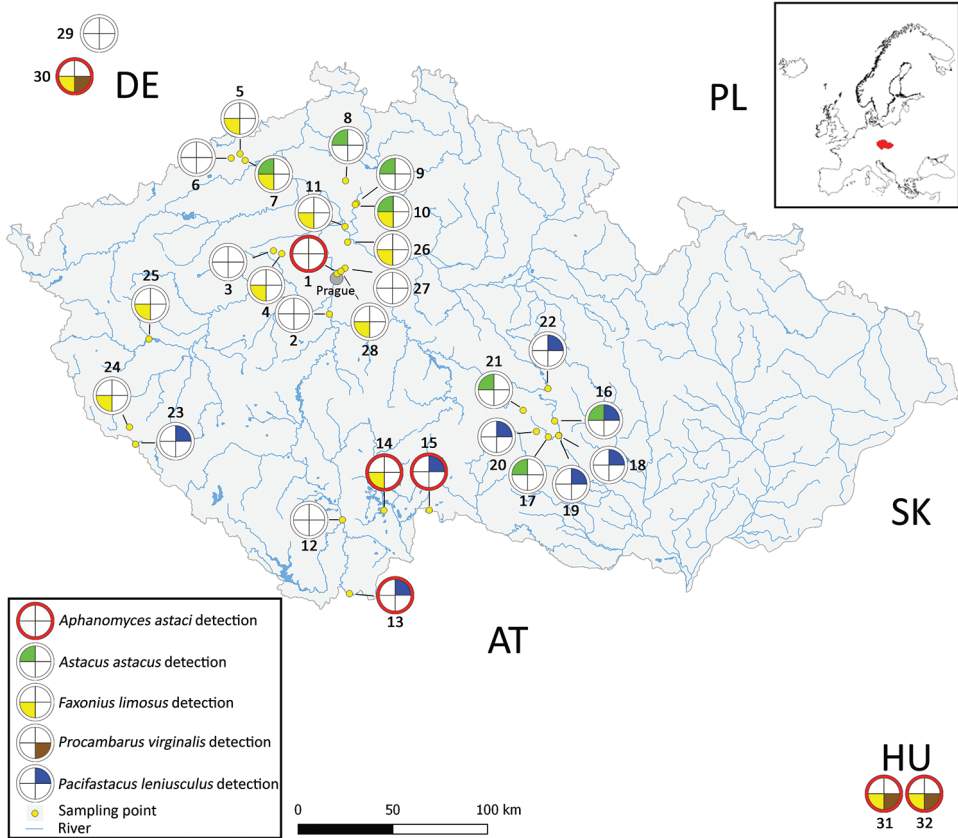


Figure 3. Map of Czechia with results of the eDNA screening at the sampling locations. Blue lines and areas represent the main water bodies, yellow dots represent each respective sampling point with numbers referring to the sampling sites in Table 2 and Suppl. material 1: Table S1. Pie charts: the red ring around the pie charts indicates unambiguous eDNA detection of *A. astaci* whereas a white ring represents non-detection. The green colour indicates detection of *A. astacus*, blue indicates detection of *P. leniusculus*, yellow represents detection of *F. limosus* and brown indicates presence of *P. virginalis*. The neighbouring countries are indicated by their two-letter ISO codes: AT, DE, HU, PL and SK stand for Austria, Germany, Hungary, Poland and Slovakia.

sampling points, except at location 16 (Oslava) where eDNA of *A. astacus* was also detected. Environmental DNA from *P. leniusculus* never co-occurred with other non-native crayfish species.

Environmental DNA of non-native *F. limosus* was unambiguously detected in 13 locations. At one location the detection occurred on only one filter. *In-situ* observation on the day of sampling confirmed the eDNA results at eight locations. Environmental DNA from the crayfish plague pathogen *A. astaci* was detected in four of the *F. limosus*-positive locations, three of which were urban waters of Berlin (site 30 – Hundekehlesee) and Budapest (31 and 32 – Barát); presence of infected crayfish was confirmed at site no.

Table 2. Results of the eDNA analyses from individual sampling sites. Volumes of water filtered (in l) indicated. The target species are abbreviated as follows: AA for *Astacus astacus* (noble crayfish), PL for *Pacifastacus leniusculus* (signal crayfish), PV for *Procambarus virginalis* (marbled crayfish), FL for *Faxonius limosus* (spiny-cheek crayfish) and Aph for *Aphanomyces astaci* (crayfish plague agent). The column labelled “obs” indicates any crayfish observed at the respective site during the sampling, using the same species abbreviations. Sites where manual search for crayfish was impossible to conduct are indicated by “ns”. Detection in eDNA samples is stated as unambiguous confirmation on 0 (marked as “–”), 1 or 2 filters per site (for more details, see Suppl. material 4: Table S4). The prevalence of *A. astaci* in NICS populations and maximum agent level in infected crayfish following Vrålstad et al. (2009) is specified. For more details about the sampling sites and specific comments, including past evidence of crayfish presence, see Suppl. material 1: Table S1.

No.	Locations	Habitat	Volume (in l)	qPCR positives in eDNA samples						<i>A. astaci</i> screening in NICS	
				AA	PL	FL	PV	Aph	obs	Prevalence	Max. agent level
1	Vltava in Prague	River	4	–	–	–	–	2		88% (15/17)	A4
2	Vltava (Vrané)	Reservoir	2.2	–	–	–	–	–		n/a	
3	Kněžák Pond	Fishpond	1.35	–	–	–	–	–		n/a	
4	Smečno	Urban pond	1.9	–	–	1	–	–		n/a	
5	Barbora	Flooded mine	10	–	–	2	–	–	FL	0% (0/22)	(3 x A1)
6	Osecký Pond	Fishpond	0.7	–	–	–	–	–		n/a	
7	Bouřlivec (Všechlapy)	Reservoir	2.8	1	–	2	–	–	ns	n/a	
8	Liběchovka	Stream	1.5	2	–	–	–	–		n/a	
9	Pšovka (above Harasov)	Stream	4.4	2	–	–	–	–	AA	n/a	
10	Pšovka (Harasov)	Pond out	10	1	–	2	–	–	FL	20% (3/15)	A2
11	Elbe	River	3.8	–	–	2	–	–	FL	35% (6/17)	A4
12	Malše in České Budějovice	River	1.85	–	–	–	–	–		n/a	
13	Malše (border with Austria)	Stream	10	–	2	–	–	2	PL	80% (16/20)	A3
14	Zlatá stoka	Channel	1.6	–	–	2	–	1		12.5% (1/8)	A3
15	Dračice	Stream	1.2	–	2	–	–	2	PL	100% (20/20)	A5
16	Oslava (upstream)	Stream	2.3	2	2	–	–	–	PL	0% (0/23)	A0
17	Balinka (upstream)	Stream	4	2	–	–	–	–	PL	n/a	
18	Oslava (confluence)	Small river	10	–	2	–	–	–	PL	n/a	
19	Balinka (confluence)	Stream	4.1	–	2	–	–	–		n/a	
20	Žďárka	Stream	5.1	–	2	–	–	–	PL	0% (0/28)	A0
21	Ochozský Brook	Stream	0.85	2	–	–	–	–	AA	n/a	
22	Staviště	Stream	4.4	–	2	–	–	–	PL	0% (0/18)	A0
23	Kouba	Stream	3	–	2	–	–	–	PL	n/a	
24	Starý Klíčov – Lomeček	Quarry	10	–	–	2	–	–	ns	n/a	
25	Mže (Hracholusky)	Reservoir	3.2	–	–	2	–	–	FL	29% (2/10)	A3
26	Kojetice	Quarry	10	–	–	2	–	–	FL	70% (14/20)	A2

No.	Locations	Habitat	Volume (in l)	qPCR positives in eDNA samples						<i>A. astaci</i> screening in NICS	
				AA	PL	FL	PV	Aph	obs	Prevalence	Max. agent level
27	Prague–Prosek (park)	Urban pond	10	–	–	–	–	–		n/a	
28	Rokytká	Stream	2	–	–	2	–	–		n/a	
29	Krumme Lanke	Lake	10	–	–	–	–	–	ns	n/a	
30	Hundekehlesee	Lake	10	–	–	2	1	1	ns	n/a	
31	Tributary of Barát	Thermal stream	10	–	–	2	2	2	FL, PV	85% (17/20)	A3
32	Barát Brook	Stream	10	–	–	2	2	2	FL, PV	n/a	

31. In four locations (10 – Pšovka, 11 – Elbe, 25 – Mže and 26 – Kojetice), data from *F. limosus* tissue analyses confirmed *A. astaci* prevalence ranging from low to high (20%, 35%, 29% and 70% respectively) and very low to moderate infection load (A2, A4, A3 and A2), but no *A. astaci* spores were detected by eDNA there. Environmental DNA of *F. limosus* and native *A. astacus* was detected together in two locations (mentioned above; Table 2, Fig. 3). *Faxonius limosus* eDNA did not co-occur with that of other non-native crayfish species in Czechia, but did so at both locations in Budapest (31 and 32) and one location in Berlin (30 – Hundekehlesee) (Table 2, Fig. 3). These three urban waters were the only sites where we confirmed eDNA of *P. virginalis* (in all cases together with *A. astaci*). Specimens of this crayfish species were observed at the Hungarian sampling sites.

In 24 subsamples (i.e. technical replicates), eDNA of *A. astaci* was detected (with Ct values in the qPCR reaction not exceeding 41; Suppl. material 4: Table S4), but it was quantifiable only in 12 subsamples. Four of these detections (33%) showed inhibition, mostly weak. *Astacus astacus* eDNA was detected in 27 subsamples of which 17 were above the LOQ. Two of these (12%) displayed weak inhibition. All of the 32 subsamples that were positive for *P. leniusculus* were quantifiable and none of them showed any inhibition. Of 49 subsamples positive for *F. limosus*, ten were quantifiable (above LOQ) and four (40%) showed some inhibition. Ten subsamples were positive for *P. virginalis* of which eight were quantifiable; Δ Ct values for these subsamples indicated some qPCR inhibition as well.

Discussion

Crayfish eDNA and assays – our study compared to the state of art

This study explores the use of the eDNA methodology for the detection of the crayfish plague pathogen *A. astaci* and freshwater crayfish in Central and Western Europe, simultaneously covering several species and numerous habitat types. A steadily increasing number of studies use eDNA monitoring to assess the presence of native crayfish or the introduction and spread of non-native crayfish across the globe (Tréguier et al. 2014; Dougherty et al. 2016; Ikeda et al. 2016, 2019; Agersnap et al. 2017; Larson

et al. 2017; Mauvisseau et al. 2018). In Europe, these tend to be complemented by screening for the accompanying conservationally relevant pathogen *A. astaci* (Robinson et al. 2018; Mauvisseau et al. 2019; Strand et al. 2019; Wittwer et al. 2019).

One of the potential pitfalls of eDNA monitoring methods, relying on species-specific qPCR, lies within the development and testing of the assays themselves. Specificity testing, both *in silico* and *in vitro* against isolates of any closely-related species that may cause false-positive results, is therefore imperative. While several previous studies have performed specificity testing on a limited range of locally relevant freshwater crayfish species (Dougherty et al. 2016; Agersnap et al. 2017) and one on a more comprehensive range of non-target species than just those found in the examined area (Larson et al. 2017), we tested the assays used for *A. astacus*, *P. leniusculus*, *P. virginalis* (this study) and *F. limosus* (Mauvisseau et al. 2018) towards most native and non-native freshwater crayfish species known from European waters (Suppl. material 2: Table S2). The three former assays proved sufficiently specific, although a weak cross-amplification with other cambarids was observed when testing the *P. virginalis* assay against DNA isolates from other crayfish. However, the *F. limosus* assay yielded a relatively strong non-target amplification for *F. virilis* with the originally recommended annealing temperature (56 °C). An increase of the annealing temperature to 60 °C reduced its extent, but DNA of several other *Faxonius* and *Procambarus* species also yielded cross-amplification with this assay. We may presume that at 56 °C this effect would be substantially stronger.

The cross-amplification of non-target species at high Ct levels, close to cut-off of both assays for *F. limosus* and *P. virginalis*, should pose no practical problems in eDNA studies, as these were observed while analysing tissue isolates. Environmental samples contain, by their very nature, less DNA of the target species than tissue isolates and thus usually amplify more than 10 cycles later compared to DNA isolates from tissue. A false-positive detection is therefore highly unlikely to occur for most of these taxa, possibly with the exception of *F. virilis* detection by the *F. limosus* assay. Yet, it seems that achieving universal specificity for assays may pose a challenge, especially in regions with higher crayfish species biodiversity than Europe where closely-related species can co-occur that differ only marginally in the target DNA marker. In such cases it may be beneficial to apply the metabarcoding approach with general primers to better capture the overall crayfish biodiversity (Thomsen et al. 2012a).

However, for management purposes in Europe, even the non-specific amplification of *F. virilis* is not likely to pose a substantial problem as non-native *F. virilis* has so far only been found in London (Ahern et al. 2008) and the Netherlands (Soes and van Eekelen 2006). Moreover, even in the case of such a false detection, this still indicates the presence of an invasive crayfish of concern to the EU (Regulation (EU) No 1143/2014) that may act as a crayfish plague carrier (Tilmans et al. 2014).

Environmental DNA monitoring of crayfish – pros and cons

An increasing number of studies, including the present one, demonstrate that the eDNA approach is effective in providing presence/absence data for freshwater crayfish

(Dougherty et al. 2016; Ikeda et al. 2016, 2019; Agersnap et al. 2017; Mauvisseau et al. 2018, 2019; Strand et al. 2019). In contrast to the crayfish plague agent *A. astaci*, where it is possible to determine the rough quantity of spores in the water (Strand et al. 2011, 2012, 2014; Makkonen et al. 2013; Svoboda et al. 2013, 2014), it is not possible to quantify crayfish biomass, population density or population structure on the basis of eDNA detection (Dougherty et al. 2016; Agersnap et al. 2017; Laurendz 2017; Rice et al. 2018).

For conservation purposes, for example when determining the suitability of an unpopulated habitat as an ark site, the critical information is nevertheless the presence or absence of the crayfish plague pathogen and any potential vectors thereof. For this purpose, eDNA monitoring provides an efficient alternative for confirming the presence of target organisms (Strand et al. 2019). However, caution must be exercised regarding the interpretation of samples that do not yield any positive detection. Many samples and large volumes should be analysed to substantiate the high likelihood of absence of a rare target organism convincingly (Strand et al. 2014, 2019).

In this study, we failed to detect *A. astaci* eDNA in four of eight locations where crayfish tissue analyses confirmed the presence of this pathogen, albeit in either a low prevalence or low infection load. Here, we have no knowledge about the density of the carrier-population, but the combination of low pathogen prevalence and low crayfish population density is obviously a challenge to reveal *A. astaci* presence in a random water sample. At location 29 (Krumme Lanke), we were unable to detect eDNA of any of the five target organisms despite reports of the presence of both *F. limosus* and *P. virginalis* somewhere in the lake in the recent past (Linzmaier et al. 2018). This might be explained by spatial mismatch (Harper et al. 2018) and low ambient temperatures which may have led to decreased activity of crayfish (Bubb et al. 2004; Rusch and Füreder 2015) and thus decreased emission of eDNA.

Dilution of the eDNA amount in large waterbodies is a factor that may lead to the failure to detect the target taxa, even if present. This is also exemplified in location 1 (the river Vltava in Prague) where we detected the crayfish plague agent but none of the host species. At this sampling site, the Vltava is more than 115 m wide and the flow rate on the date of sampling was $\sim 50 \text{ m}^3/\text{s}$, so any eDNA signal would be subject to significant dilution, a common problem reported in previous studies (Strand et al. 2014, 2019). The presence of *F. limosus* in the Vltava in Prague has previously been confirmed, with crayfish displaying high levels of infection with *A. astaci* (Table 2) only a short distance downstream from the sampling site. Furthermore, *A. astaci* spores are alive and active and will more likely withstand chemical and biological processes in the water that lead to degradation of eDNA (Laurendz 2017), compared to cells shed from crayfish, a group reported to release only a very low amount of eDNA (Rice et al. 2018).

Strand et al. (2019) monthly monitored a watercourse for more than a year during an ongoing crayfish plague outbreak in Norway. There, the very scarce population of *P. leniusculus* that had caused the plague outbreak was detected by eDNA only in July and October, concurring with the presumed periods of moulting and reproduction, when more eDNA from the crayfish is likely to be released to the water. Dunn et al.

(2017) examined the relationship between eDNA concentration and crayfish biomass and were able to detect a relationship only when female *P. leniusculus* crayfish were ovigerous. Laurendz (2017) found no clear correlation between number of crayfish and eDNA emission in aquaria experiments with *P. leniusculus*, but observed peaks during moulting and huge quantitative variation depending on various environmental and biological factors. Similarly, Buxton et al. (2017) observed peaks of eDNA of the great crested newt (*Triturus cristatus*) towards the end of the adult breeding period and when newt larval abundance was at its highest. While studying seasonal variation of eDNA emission by freshwater pearl mussel (*Margaritifera margaritifera*), Wacker et al. (2019) measured the highest concentrations of eDNA in August, corresponding to the period these mussels release large amounts of larvae into the water. These studies and our results demonstrate that sample number, coverage, season, inhibition and other environmental factors can substantially influence the results and that eDNA methods may fail to detect elusive or rare targets. A robust knowledge of the biology of the target species is thus required for improving sampling success. In our study, although using large volumes that to some degree compensate for few samples, we would most likely increase the detection success with more samples.

A useful tool to help determine the number of samples required for maximising detection probability could be occupancy modelling. Schmidt et al. (2013) analysed data obtained while examining the presence of the chytrid fungus *Batrachochytrium dendrobatidis*. Based on an index similar to “catch-per-unit-effort”, which is also obtainable for crayfish, they were able to calculate the amount of samples required for a detection probability to exceed 95%. Dougherty et al. (2016) used relative abundance and site characteristics as covariates to model the detection probability for *F. rusticus* using eDNA sampling. A similar tool for occupancy modelling, an R package for multiscale occupancy modelling of eDNA data, was recently presented by Dorazio and Erickson (2017).

Detection of the host-pathogen complex

In the screening of crayfish habitats, we successfully managed to detect eDNA of European noble crayfish and all three North American crayfish species investigated in this study. Here, we infrequently detected eDNA of the crayfish plague pathogen *A. astaci* together with the three investigated non-native crayfish species. More commonly, only eDNA from non-native crayfish was detected alone, suggesting low prevalence and infection load or possibly even absence of the pathogen (as also corroborated by analyses of the host crayfish tissues).

The eDNA monitoring methodology has been promoted as a reliable, non-invasive, ethical and animal welfare-friendly alternative to cage monitoring for early detection of crayfish plague (Wittwer et al. 2017; Strand et al. 2019). Indeed, when eDNA fails to detect *A. astaci*, although present at the location, it is likely that the pathogen spore concentration is too low to infect caged susceptible crayfish anyway. Strand et al. (2019) demonstrated that eDNA monitoring reveals the presence of *A. astaci* in the water earlier

than cages with live crayfish put out for disease surveillance. According to Strand et al. (2019), the simultaneous monitoring of native and non-native crayfish also provides additional information on habitat status, which otherwise requires trapping surveys.

We never detected eDNA from *A. astaci* together with native *A. astacus*, which is a good sign for the habitat status for these locations. However, in a few locations, eDNA from both native and non-native crayfish co-occurred. This could, in some cases, result from passive downstream transport of eDNA (Deiner and Altermatt 2014; Rice et al. 2018) from one of the target species that was geographically separated – even with migration barriers. However, in other cases it could reflect co-existence of native and non-native crayfish in the absence of infected crayfish carriers, or with very low *A. astaci* prevalence in the non-native crayfish population. In the latter case, it might only be a matter of time before the low-prevalent crayfish plague agent eradicates the native population. In a Norwegian lake, populations of *A. astacus* and *A. astaci*-carrying *P. leniusculus* presumably occurred at the same time for more than a decade before crayfish plague struck the native population (Vrålstad et al. 2011, 2014). This might be explained by low infection pressure and geographic separation within the lake.

The observed co-occurrence of eDNA from *A. astacus* and *F. limosus* in two locations, as well as *A. astacus* and *P. leniusculus* in one location, could suggest a possible syntopic presence of native and non-native species, although in at least one of the cases (location 10), downstream transport of *A. astacus* eDNA from a population upstream of the *F. limosus* population (location 9) is more likely. However, co-existence can occur in the absence of *A. astaci* infection in the non-native population. This has been thoroughly documented in Central Europe for *F. limosus* populations co-occurring with *A. astacus* (Schrimpf et al. 2013) and also for *P. leniusculus* populations co-occurring with *A. astacus* in Denmark (Skov et al. 2011). In our study, 70% and 80% of the *P. leniusculus* and *F. limosus* locations did not yield positive eDNA results for *A. astaci*, respectively. However, the number of individuals directly tested by us for infection was too low to conclude about the absence of the pathogen even at places where none was detected (see Schrimpf et al. 2013).

The co-occurrence of NICS in urban waters, represented by an inner-city lake (30 – Hundekehlensee) and a thermal stream (31 and 32 – Barát stream and its thermal inflow), demonstrates the importance these habitats play for the spread of NICS. The ornamental pet trade has been shown to be a major introduction pathway for non-native crayfish species into Europe (Peay 2009b; Chucholl 2013) and the species found at these locations are available through the pet trade (Mrugała et al. 2015). Additionally, eDNA of the crayfish plague pathogen *A. astaci* was detected at all three locations. Our findings highlight both the risks emanating from these habitats as well as the possibilities of monitoring similar habitats using eDNA.

Methods and sample strategies

The use of eDNA plays an important role in the present efforts to introduce advanced molecular tools into monitoring and bio-assessment of aquatic ecosystems (Leese et

al. 2016). This is particularly important with regard to the protection, preservation and restoration of aquatic ecosystems, which for European Union countries is legally binding through the Water Framework Directive (EU directive 2000/60/EC). Current approaches are still largely based on traditional sampling of organisms followed by identification by morphology, which is time-consuming and error-prone due to the varying and diminishing taxonomic expertise (Leese et al. 2016). While metabarcoding of environmental samples is the most promising approach for bioassessment and biodiversity inventory studies (de Vargas et al. 2015; Visco et al. 2015; Fujii et al. 2019), the more targeted qPCR approaches are specifically relevant for the monitoring of rare and red-listed native species and/or harmful invasive species of particular focus.

For both approaches, sampling strategies are of great importance for the quality and outcome regarding results. The choice of sample method, filter and volume might be of vital importance for maximising the detection probability of rare targets (Strand et al. 2014; Kumar et al. 2019). Crustaceans are more challenging to detect (Forsström and Vasemägi 2016; Rice et al. 2018) than fish, for example, that shed multiple sources of eDNA into the water (Jo et al. 2019). It appears, therefore, that efficient eDNA sampling for crayfish and their pathogen requires a substantially larger volume of water than for fish and amphibians. However, we are not aware of any study directly comparing these organisms.

The cost of the sampling equipment, as used for example in Strand et al. (2014, 2019) or Thomas et al. (2018), may be a limiting factor that prevents collection of suitable samples by a wider body of stakeholders. While conducting the fieldwork, we thus also evaluated the applicability of a robust, easy-to-use and low-cost version of the eDNA sampling equipment, based mostly on items readily available in garden stores and hobby markets. Most importantly, we exchanged the costly Masterflex E/S portable peristaltic pump-based sampler (retail price exceeding 2000 USD) with the drill-powered pumping system (ca. 26 USD without drill). This low-cost alternative provided very satisfactory results since it was possible to filter the same amount of water as sampled with the Masterflex E/S sampler and the target organisms were usually detected where expected. The difference between the two systems, which use exactly the same filter, is that water is pumped through the filter with suction, rather than pressure, since the filter is situated at the front of the drill-pump system. All parts of the entire setup can be detached and disinfected and the easy-to-replace filter cups eliminate issues with potential carry-over contamination. The low price of the equipment is a particularly important benefit for various stakeholders with limited budgets (e.g. nature conservancy agencies, NGOs, fishery managers).

Compared to the traditional methods used to determine presence or absence of crayfish which consist of either manual searching or trapping, this method requires less time in the field at each sampling site and it allows for sampling at locations unsuitable for traditional monitoring. For example, some of the sampling points visited by us were inaccessible for manual searching crayfish and would have required trapping or scuba diving, neither of which was possible during the fieldwork for this study. The eDNA methodology also enables the user to detect crayfish species when only small-sized in-

dividuals which might neither be caught in traps nor easily detected by manual search are dominant. Additionally, the extracted eDNA filter samples contain a broad variety of species from each location, both microorganisms and macroorganisms, and can be, at a later date, screened for entirely different targets (Dysthe et al. 2018). There is thus a potential for savings of both effort and costs if relevant stakeholders synchronise and/or collaborate on the eDNA sampling for multiple research and monitoring purposes.

Conclusions

The eDNA method based on targeted species-specific qPCR is suitable for detecting several invasive and native crayfish species as well as the crayfish plague pathogen in relevant habitat types in Central and Western Europe. The assays presented here performed well and yielded results that mostly corroborated our knowledge on the presence of native and non-native crayfish in the visited habitats.

It is particularly the positive data on the presence of crayfish and crayfish plague that yield valuable information, while negative results have to be interpreted with great caution. The latter should preferably be followed up with analyses of more samples collected in suitable periods, taking into account the time of year, temperature, water flow and the biology of the target species. This is of paramount importance if the absence of a specific species needs to be unambiguously established.

Including further assays of other crayfish species native to Central Europe, such as the stone crayfish, into this already broad panel will enable relevant stakeholders and authorities to use this method as a routine monitoring tool for all relevant crayfish species or in preparation of restocking operations.

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Supplementary material 1

Table S1

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: details on localities

Explanation note: Detailed information about the eDNA sampling sites visited during the study.

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Link: <https://doi.org/10.3897/neobiota.58.49358.suppl1>

Supplementary material 2

Table S2

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: species list

Explanation note: List of crayfish species used for in-vitro testing of the assay specificity.

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Link: <https://doi.org/10.3897/neobiota.58.49358.suppl2>

Supplementary material 3

Table S3

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: data for methods

Explanation note: Standard dilutions from crayfish genomic DNA.

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Link: <https://doi.org/10.3897/neobiota.58.49358.suppl3>

Supplementary material 4

Table S4

Authors: Johannes C. Rusch, Michaela Mojžišová, David A. Strand, Jitka Svobodová, Trude Vrålstad, Adam Petrusek

Data type: detailed results

Explanation note: Overview of the qPCR results, cDNA copy number estimation and PCR inhibition.

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Link: <https://doi.org/10.3897/neobiota.58.49358.suppl4>

Perceptions of alien plants and animals and acceptance of control methods among different societal groups

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Abstract

Biological invasions are a widespread phenomenon and cause substantial impacts on the natural environment and human livelihoods. Thus, the European Union (EU) recently adopted Regulation No 1143/2014 to limit the negative impacts of invasive alien species (IAS). For implementing IAS management and policies, public support is highly and increasingly important, especially when it comes to charismatic species and lethal methods. Recognising the importance of the interaction of public perception with acceptance of IAS management methods, we used an online survey targeting three different stakeholder groups in Austria to evaluate potential differences in perception of IAS and management methods.

In total, we received 239 completed responses: 20 nature users (farmers, hunters), 91 nature experts (conservationists, biologists) and 128 from the general public. Participants were more likely to accept lethal management methods when it was an IAS. Nature experts' acceptance of IAS management methods was rather similar to those of nature users, while the general public preferred non-lethal methods. Chemical lethal methods (herbicides, poison pellets) received low rates of acceptance throughout all stakeholder groups, although nature users were more open to accept such methods for plants. Most respondents (> 50%) were not aware of the role of the EU in IAS topics nor did they know of the existence of the EU IAS regulation 1143/2014. However, more than 75% of respondents agreed that IAS measures and regulations should be implemented at EU level.

This study shows that knowledge about native versus invasive alien status has an influence on the acceptance of management methods. Nature users may have higher levels of acceptance of lethal methods because they are economically dependent on extracting resources from nature. Invasive alien species regulations on EU level are generally acceptable, but there is low awareness for actions already undertaken EU.

Keywords

EU Regulation 1143/2014, IAS control, management methods acceptance, nature conservation, perception, survey, values

Introduction

Biological invasions are a widespread phenomenon and cause substantial impacts on the natural environment and human livelihoods (Pejchar and Mooney 2009; Villà et al. 2011). Thus, one of the targets of the EU Biodiversity Strategy 2020 (European Commission 2011) is to “combat invasive species”, i.e. to halt – or at least to reduce – the negative impacts caused by biological invasions. To achieve this, “Regulation (EU) No 1143/2014 on the prevention and management of the introduction and spread of invasive alien species [IAS]” was adopted in 2014 (European Parliament 2014).

According to this regulation, “alien species” are defined as any live specimen of a species, subspecies or lower taxon of animals, plants, fungi or micro-organisms introduced outside its natural range; it includes any part, gametes, seeds, eggs or propagules of such species, as well as any hybrids, varieties or breeds that might survive and subsequently reproduce. “Invasive alien species” are those alien species whose introduction or spread threatens or adversely impacts upon biodiversity and related ecosystem services (European Parliament 2014; Essl et al. 2018).

One key component of the EU regulation is the “List of Invasive Alien Species of Union Concern” (Roy et al. 2019). The European Commission and EU member states can propose candidate species for this list. Such candidate species have to undergo a risk assessment and, subsequently, a decision whether to include these species into the list of IAS of EU concern is made by the EU member states. Once approved, the listed species fall in the range of measurements of Regulation no. 1143/2014. Initially, this list contained 23 plant species and 26 animal species (European Commission 2016), but it has been expanded by another 16 species in spring 2019 (European Commission 2019). Generally, there are no specified recommendations for management measures in Regulation no. 1143/2014, but it is stated in Article 19 that “lethal and non-lethal physical, chemical and biological actions aimed at the eradication, population control or containment of a population of an invasive alien species” (European Parliament 2014) should be taken into consideration. Article 25 specifies that IAS management “should be proportional to the impact [of IAS] on the environment” and the operator “should take the necessary measures to spare avoidable pain, distress and suffering of animals during the process”. Moreover, “non-lethal methods should be considered and any action taken should minimise the impact on non-targeted species” (European Parliament 2014).

Therefore, the question arises if killing a charismatic animal for conservation purposes is justified and appropriate (Jaric et al. 2020). Scientific and general public opinions can differ tremendously (Bertolino and Genovesi 2003) and public opinion

becomes increasingly important for IAS management (Vaske et al. 2011; Verbrugge et al. 2013; Crowley et al. 2017). A recent example in this context is the Australian feral Brumby horse. In 2018, public pressure led to the termination of scientifically-recommended conservation actions (NSW Government Office of Environment & Heritage 2016; Australian Academy of Science 2018), in this case shooting the feral horses. The government of New South Wales followed the public protesters' and animal rights activists' demand and protected an invasive alien horse (Brumby, *Equus caballus*) (Parliament of New South Wales 2018), although scientific evidence shows that Brumbies threaten habitats and native species (Nimmo and Miller 2007; Worboys and Pulsford 2013; NSW Threatened Species Scientific Committee 2016). A similar situation unfolded in Italy in the late 1990s, when animal rights activists stopped the eradication of a population of invasive grey squirrels (*Sciurus carolinensis*), which is native to eastern North America, by taking the responsible conservation institute to court (Bertolino and Genovesi 2003). These examples raise the question as to what underlies the motivations that cause public opposition to population reduction measures of invasive alien species.

Aesthetic and charismatic species are often used as flagship species for engaging stakeholders, increasing acceptance and promoting conservation programmes (Caro and Girling 2010). Cultural ecosystem services, such as aesthetic appreciation or recreation, are highly valued by people across all societies (WHO 2005). Thus, the (invasive alien) species' appearance might be one of the underlying motives for rejecting or accepting a specific management method. Further, different economic interests, value systems, preferences and biases may affect social perceptions of IAS and of management measures (Kueffer 2017; Kapitza et al. 2019; Shackleton et al. 2019a).

Here, we used an online survey targeted at three stakeholder groups. Participants assessed pairs of IAS included in the EU IAS regulation and native species. By doing so, we addressed the following questions: 1) What are the differences in perceptions of invasive alien plant and animal species and similar native species? 2) What is the level of knowledge in identifying invasive alien and native species? 3) What are the differences in acceptance of different management measures? 4) Which institutions should play stronger roles in IAS management?

Methods

Survey and sampling design

For this research, the non-probability method of self-selective convenience sampling was chosen, i.e. there are no rules for selecting the potential participants (Saunders et al. 2009). In the handbook of web-surveys (Bethlehem and Biffignandi 2012), it is defined that "elements are drawn for such a sample because of their convenient accessibility or proximity to the researcher. Convenience sampling is fast, simple and cheap. Self-selection samples can be considered a form of convenience sampling"

(Bethlehem and Biffignandi 2012). For a survey that includes large target groups, this is considered to be an appropriate approach. Our approach was partly self-selective, because it was distributed via pre-selected media channels (e.g. Facebook-posting, E-mail).

An additional advantage of convenience sampling is that it facilitates reaching out to participants from stakeholder groups that are otherwise difficult to reach (Saunders et al. 2009), for example, via selected media. The main disadvantages of this method is that results cannot easily be generalised to the entire stakeholder group (Bethlehem and Biffignandi 2012; Raab-Steiner and Benesch 2018; Schnell et al. 2018). In addition, response rates cannot be calculated accurately (Bethlehem and Biffignandi 2012). However, the demographic data of the participants are helpful for interpreting the results and for identifying potential biases in participation.

We designed an online survey (in German; see Suppl. material 1: Text S1 for German and Suppl. material 1: Text S2 for translated English version) which was circulated widely to potential participants in Austria. The online survey used species pairs consisting of native – invasive alien species (Figure 1). The survey had nine questions that referred to these species pairs and which dealt with i) perception, ii) management measures, iii) knowledge on native/invasive alien status, iv) relevance of invasive alien species to Austrian biodiversity and environmental management and iv) knowledge and performance of EU IAS policies. A Likert-type-scale approach was chosen for all questions, which captured the response of the recipients depending on the dis-/agreement to the respective statement (Likert 1932; Raab-Steiner and Benesch 2018).

Study species selection and description

A total of four species pairs (thus eight species in total) consisting of a native and an invasive alien species were selected. We used two mammal species pairs and two vascular plant species pairs. The four invasive alien species are included in the “EU List of IAS of Union Concern” (European Commission 2016). The invasive alien species were paired with species native to Austria which have a similar physical appearance (Figure 1) and occur in similar habitats (Tables 1, 2). Each study species was shown by one photograph. To maximise comparability among species, photos were selected to show one adult individual of the study species (for mammals) or a population in full flower (for plants). Moreover, we selected photos that show species in similar situations (Figure 1).

Scope and questions of the survey

Perception of species

For every study species pair, the species photos were shown together with six questions which referred to the attitude of the survey participant towards the species.





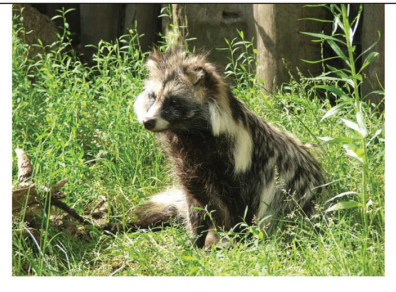



Invasive alien species	Native species
	
a) Common Milkweed (<i>Asclepias syriaca</i>)	b) White Swallow-Wort (<i>Vincetoxicum hirundinaria</i>)
	
c) Himalayan Balsam (<i>Impatiens glandulifera</i>)	d) Touch-me-not-Balsam (<i>Impatiens noli-tangere</i>)
	
e) Raccoon Dog (<i>Nyctereutes procyonoides</i>)	f) Red Fox (<i>Vulpes vulpes</i>)
	
g) Raccoon (<i>Procyon lotor</i>)	h) Beech Marten (<i>Martes foina</i>)

Figure 1. Photos of the four pairs of native versus invasive alien study species used in the online survey. All photos are from Wikimedia Commons **a** Анимална хикари **b** H. Zell **c** Donald Hobern **d** Malte **e** I. Pkuczynski **f** <http://www.nps.gov/acad/photos/redfox.htm> [Public domain] **g** Bastique **h** Zefram.

Table 1. Overview of the distribution, region of origin, first records and habitat affiliation of the invasive alien species of the survey (Essl and Rabitsch 2002; Umweltbundesamt 2019).

	Common Milkweed (<i>Asclepias syriaca</i>)	Himalayan Balsam (<i>Impatiens glandulifera</i>)	Raccoon Dog (<i>Nyctereutes procyonoides</i>)	Raccoon (<i>Procyon lotor</i>)
Origin	North America	India, Himalaya	Siberia, Ussuri, Manchuria, Korea, Japan	North and Central America
Habitat	ruderal slopes, roadsides, fallow	riversides, floodplains, ruderal plains, wet fallows	broadleaf and mixed forests, near waterbodies	wet broadleaf forests, near waterbodies, near settlements
Distribution in Austria	Vienna, Lower Austria, Upper Austria, Styria, Burgenland, Carinthia	All of Austria	Lower Austria, Upper Austria, Styria, Burgenland, Salzburg	Vienna, Lower Austria, Upper Austria, Styria, Carinthia, Vorarlberg, Salzburg
Ecological impacts	displacement of native plants; overgrowing of large areas; high spreading	displacement of native plants (riverside vegetation); overgrowing of large areas; high spreading	predation of molluscs, insects and amphibians; transmitter of diseases	no detailed data, predation of bird nests, amphibians, reptilians and fish; transmitter of diseases
First record in Austria	unknown	1898	1963	1974

Table 2. Overview of the distribution and habitat affiliation of the native species of the survey (Bellmann 2015; Fischer et al. 2005).

	White Swallow- Wort (<i>Vincetoxicum hirundinaria</i>)	Touch-me-not-Balsam (<i>Impatiens noli-tangere</i>)	Red fox (<i>Vulpes vulpes</i>)	Beech Marten (<i>Martes foina</i>)
Habitat	dry grasslands, open forests	riversides, floodplains, tall herb vegetation, broadleaf and mixed forests	cultural landscapes, settlements	parks, gardens, settlements, cultural landscapes
Distribution in Austria	widespread	widespread	widespread	widespread

Management methods

For each study species, a list of different management methods was presented. The participants were asked to assess the acceptance of these management methods and if there is a need to reduce or halt the spread of the species. The questions and the answer options were identical for mammal and vascular plant species.

The management methods presented were chosen according to Article 19 of Regulation (EU) no.1143/2014. As stated in the Regulation, the management option selection consists of “lethal and non-lethal physical, chemical and biological actions aimed at the eradication, population control or containment of a population of an invasive alien species” (European Parliament 2014). In addition, questions referring to relevant laws (e.g. EU IAS regulation) were included.

Species knowledge

The eight study species were shown and the participants were asked to specify for each species if it was native or invasive alien.

Topic relevance

First, the participants were asked to give an assessment of the relevance of alien species in general and their management for Austria. Subsequently, the contribution of different stakeholders (EU, national and regional governments, NGOs, farmers, foresters, hunters, gardeners, landscape architects) to IAS management was asked (five-point verbal unipolar scale, ranging from no agreement to strong agreement (Raab-Steiner and Benesch 2018)).

Awareness of EU IAS policies

As Regulation (EU) no. 1143/2014 is the cornerstone of European IAS policies, participants were asked about their awareness of this Regulation and if they believed that IAS policies indeed required an EU regulation. For these purposes, participants were asked to assess statements on the usefulness of the EU IAS legislation. Participants had the choice between “agree”, “no answer” and “disagree”.

Personal data

The following personal data of the participant was recorded: gender, age, size of municipality of residence, home country and highest completed level of education. These personal data were used to assess the characteristics of the sample of survey participants. These questions were presented with single-choice options.

Focal stakeholder groups

We selected participants from three pre-defined stakeholder groups. To do so, participants were asked to characterise themselves at the beginning of the survey as members of one of the following three stakeholder groups: i) Nature-Users (farmers, hunters, gardeners, landscape architects, foresters), b) Nature-Experts (biologists, environmental-NGO-staff, nature-conservationists) and c) General public (participants who do not belong to the above-mentioned groups). The same set of questions was used for all three groups of participants.

Survey execution

For the online survey, the software Limesurvey 3.15 (<https://www.limesurvey.org/>) was used. It was installed on a server provided by the University of Vienna. The survey was conducted in German, because the main target groups were people living in Austria. The survey was open from 5 November to 25 December 2018. The following media

outlets were used for distributing the survey: Facebook (<https://www.facebook.com/>), WhatsApp and E-mail. As the convenience sampling method was used, it was considered acceptable to choose specific media channels to reach potential participants of the different stakeholder groups. On Facebook, for example, the link to the survey was posted in different “groups” for Austrian biologists. A reminder was sent via E-mail and posted on the used social media two weeks after the first call. Several participants were contacted directly via E-mail or chat message.

Data analysis

In total, 967 participants started the survey, of which 239 (24.8%) fully completed it. Non-completed surveys ($n = 728$) were excluded from the analysis. For analyses, we pooled the responses per person (by calculating the arithmetic mean) across the two study species in each of the four focal groups “native plant species”, “native mammal species”, “invasive alien plant species” and “invasive alien mammal species”.

We used the Kruskal-Wallis-Test (Kruskal and Wallis 1952) for ordinal scaled and independent samples to identify significant differences among the answers of the stakeholder groups. It was applied for the questions on perceptions and management methods and conducted for each of the four species groups (i.e. native plants, invasive alien plants, native animals and invasive alien animals). Thus, the independent variable was the stakeholder group and the dependent variables were the coded answers for the species groups. Post-hoc, Dunn’s pairwise tests (Dunn 1961) were carried out to assess the differences for the three pairs of stakeholder groups (i.e. nature users versus nature experts, nature users versus general public and nature experts versus general public) and adjusted using the Bonferroni correction (Bonferroni 1935, 1936).

We used Wilcoxon-Tests (Raab-Steiner and Benesch 2018) to test for significant differences among species groups, i.e. native plants versus invasive alien plants and native animals versus invasive alien animals within stakeholder groups. These were applied for questions on species perception and management method acceptance. Further, we tested for significant differences among responses of the three stakeholder groups. Spearman’s Rho (Daniel 1990; Raab-Steiner and Benesch 2018) was used to assess correlations between species perception and the acceptance of management methods. For nominal variables, such as the questions regarding the EU IAS regulation and relevance of IAS, χ^2 -tests (Pearson 1900) and Fisher’s exact tests (for small case numbers) (Fisher 1970) were used to test for significant differences across stakeholder groups.

Results

Distribution of participants across stakeholder groups

Of the 239 respondents who had provided full replies, 128 participants (53.5%) were members of the “general public” (GP), 20 (8.4%) “nature users” (NU) and 91 (38.1%)

“nature experts” (NE). Unless otherwise noted, these are the sample sizes used in the analyses. The majority (72%) of the participants were younger than 30 years, 63% were female and 45% lived in a large city with more than 100,000 inhabitants. This was particularly the case for the “general public” stakeholder group. Other studies on the perception of IAS have shown similar demographic patterns (Bremner and Park 2007; Lindemann-Matthies 2016).

Survey results

Knowledge of native/invasive alien status

The assignment of the species, i.e. whether they are native or invasive alien species, was similar among the stakeholder groups (Figure 2). The majority of participants assigned the species correctly. The species assignment for mammals was correct more often than for plants, where for plants, the proportion of “I don’t know” answers was 10 to 25%.

Perception of species and management method acceptance

The response on the perceived importance of the native species for ecosystem functioning in Austria was similar across all stakeholder groups. Interestingly, native mammals were rated to be more important for ecosystem functioning than any other species group (Figure 3A). The general public rated the importance of invasive alien mammals

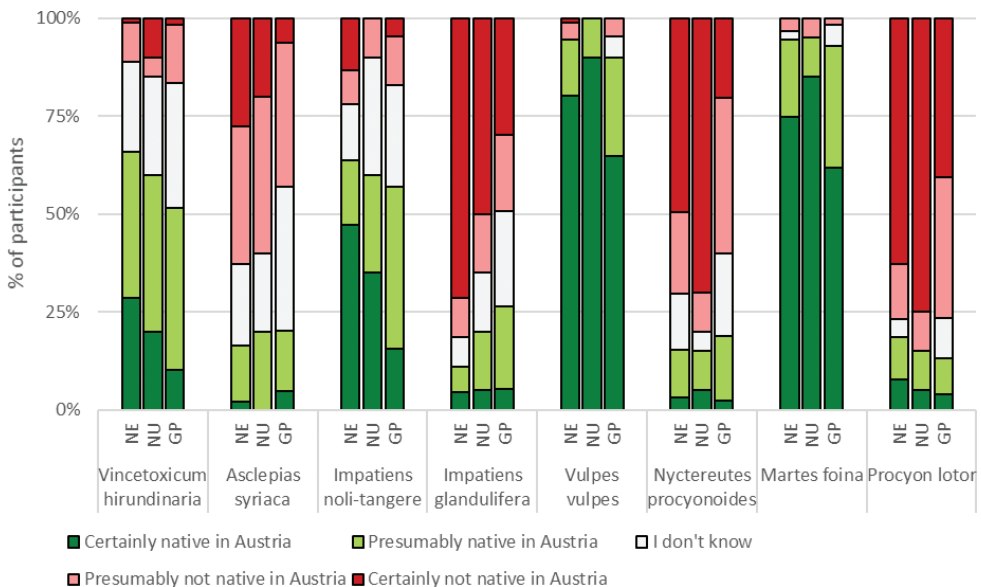


Figure 2. Participants’ knowledge about the native / invasive alien status of the study species. Abbreviations: GP = general public; NU = nature users; NE = nature experts.

for ecosystem functioning significantly better than nature experts did (Dunn-Bonferroni: $F = -2.54$; $p = 0.033$). There were significant differences between native and invasive alien plants within each stakeholder group as to the species' importance in ecosystem function (Wilcoxon tests: NE: $F = -6.46$; $p = < 0.001$, NU: $F = -1.96$; $p = 0.05$, GP: $F = -4.06$; $p = < 0.001$). Similar results were obtained for mammals (Wilcoxon tests: NE: $F = 7.31$; $p = < 0.001$, NU: $F = 3.73$; $p = 0.004$, GP: $F = 7.95$; $p = < 0.001$).

The majority of the participants across the stakeholder groups rated all species in the study as aesthetic or very aesthetic. Mammal species' aesthetics were rated higher than plant species, but this difference was not significant (Figure 3B). There were no significant differences in the rating of the aesthetics species among the three stakeholder groups. The comparison of native versus invasive alien mammal species within stakeholder groups showed that invasive alien mammals were rated significantly lower than native ones (Wilcoxon test: NE: $Z = -5.92$; $p = < 0.001$, NU: $Z = -2.91$; $p = 0.004$, GP: $Z = -4.35$; $p = < 0.001$).

The question of whether the study species belongs to Austrian ecosystems was similar among all stakeholder groups. Furthermore, native species were more frequently assigned to Austrian ecosystems than alien species (Figure 3C). The general public's answers regarding whether alien mammal species belonged to Austrian ecosystems were significantly more positive than those of the other two stakeholder groups (Dunn-Bonferroni: NU: $F = 3.28$; $p = 0.003$, NE: $F = 3.81$; $p = < 0.001$). The same was the case for alien plant species, but in this case, there is only a significant difference between nature experts and general public (Dunn-Bonferroni: $F = 4.98$; $p = < 0.001$). Within stakeholder groups, native versus invasive alien species and mammals versus plant species were rated significantly different (Figure 3D). However, the rating of native versus alien animal species was more distinct than those of native versus alien plant species.

The comparison across stakeholder groups showed that nature users had a significantly higher acceptance of clearing/shooting than the general public (Dunn-Bonferroni: IAS plants: $F = 2.55$; $p = 0.032$; native mammals: $F = 3.79$; $p = < 0.001$; IAS mammals: $F = 4.95$; $p = < 0.001$), except for native plants. The acceptance of shooting management of alien mammals varied among stakeholder groups (Kruskal-Wallis: $F = 29.94$; $p = < 0.001$) (Figure 3E). For nature experts, the acceptance level for lethal management showed significant differences between native and invasive alien species (plants: Wilcoxon: $F = 5.39$; $p = < 0.001$, mammals: Wilcoxon: $F = 4.89$; $p = < 0.001$). For nature users, clearing of invasive alien mammals was significantly more accepted than for native mammals (Wilcoxon: $F = 2.37$; $p = 0.018$). For the general public, clearing of invasive alien plants was significantly more accepted than for native plants (Wilcoxon: $F = 3.65$; $p = < 0.001$).

Nature experts' assessment of the study species belonging to Austrian landscapes (Figure 3B) and of clearing/shooting as the management method (Figure 3D) of alien species was significantly negatively correlated (mammals: Spearman-Rho = -0.56 ; $p = < 0.001$, plants: Spearman-Rho = -0.55 ; $p = < 0.001$). Participants had a lower acceptance for clearing/shooting when they assessed the species as belonging to Austrian ecosystems (Spearman-Rho = -0.39 ; $p = < 0.001$), except for native mammal species, where participants had a broad acceptance of this control method (Spearman-Rho =

0.17; $p = 0.106$). The answers to these questions by the other stakeholder groups (i.e. general public, nature users) were not significantly correlated.

Most of the participants assessed killing by chemical agents as ‘rather not’ to ‘not acceptable’ (Figure 3E). Across stakeholder groups, for alien mammal species, nature users had a significantly higher acceptance of this method than the other two groups

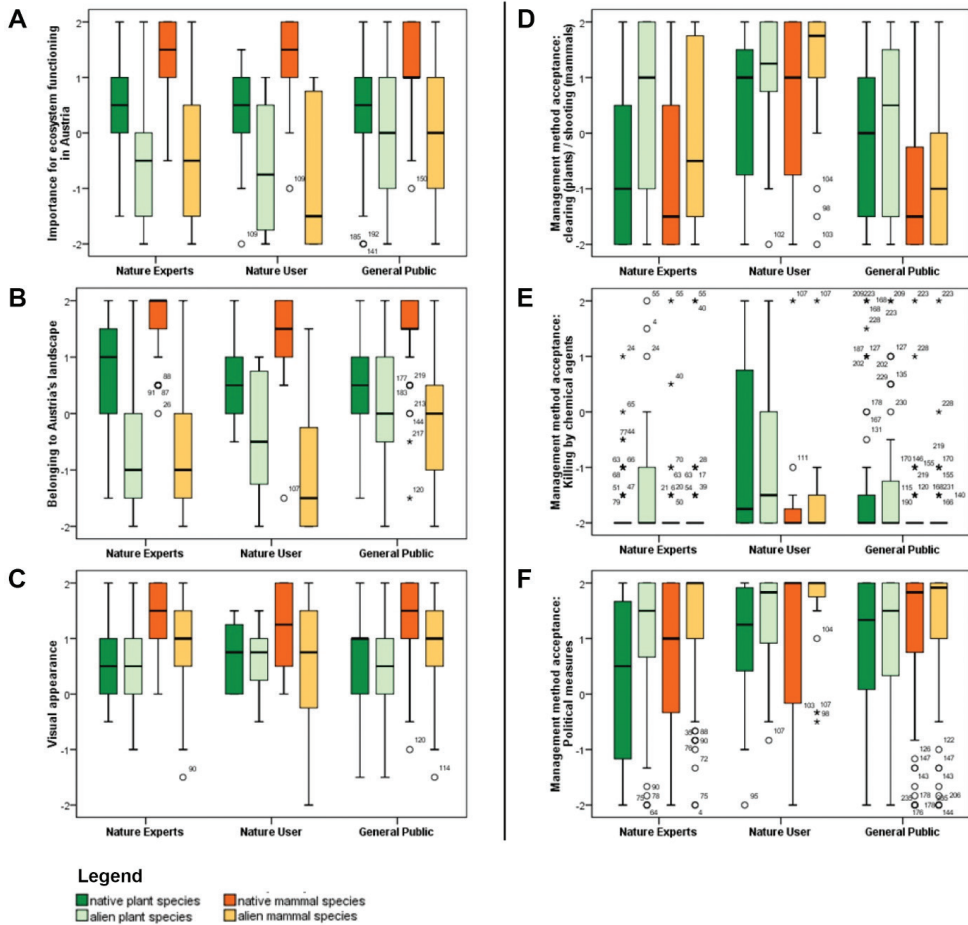


Figure 3. **A** The perceived importance of native and invasive alien plant and mammal species for ecosystem functioning in Austria rated by the three stakeholder groups. Scale: -2 (very unimportant) to 2 (very important). **B** The physical appearance of native and invasive alien plant and mammal species rated by the three stakeholder groups. Scale: -2 (not aesthetic) to 2 (very aesthetic). **C** Does the species belonging to Austrian ecosystems as rated by the three stakeholder groups. Scale: -2 (No) to 2 (Yes). **D** Assessment of the acceptance of the management method “clearing (plants)/shooting (mammals)”. Scale: -2 (not acceptable) to 2 (very acceptable). **E** Assessment of the acceptance of the management method “killing by chemical agents”. Scale: -2 (not acceptable) to 2 (very acceptable). **F** Assessment of the acceptance of the management method “legal measures” (e.g. prohibition of keeping, trading and releasing, import bans). Scale: -2 (not acceptable) to 2 (very acceptable). For significance tests, see main text.

(Kruskal-Wallis: $F = 10.91$; $p = 0.004$). The same finding applied to native mammal species, but there, the only significant difference was between nature users and the general public (Dunn-Bonferroni: $F = 2.42$; $p = 0.046$). The answers of the stakeholder groups regarding native plant species showed significant differences between nature users and nature experts (Dunn-Bonferroni: $F = 2.90$; $p = 0.011$).

Legal measures (e.g. prevention of introduction, prohibition of keeping, trading and releasing, import bans) for IAS were highly acceptable as a management method among all stakeholder groups (Figure 3F). However, nature experts' acceptance of these measures for native plant (Dunn-Bonferroni: $F = 3.21$; $p = 0.004$) and mammal species (Dunn-Bonferroni: $F = 2.85$; $p = 0.013$) was significantly lower than the general public's acceptance. The comparison of native versus invasive alien plant species within stakeholder groups showed that the acceptance for these measures was significantly higher for invasive alien plants (Wilcoxon tests: NE: $F = 5.48$; $p < 0.001$, NU: $F = 1.96$; $p = 0.05$, GP: $F = 2.93$; $p = 0.003$). The same result was found for mammals (Wilcoxon tests: NE: $F = 4.69$; $p < 0.001$, NU: $F = 2.02$; $p = 0.043$, GP: $F = 2.56$; $p = 0.01$).

Contribution of different institutions and stakeholders to IAS management

Generally, the three stakeholder groups had similar perceptions in their assessment of different stakeholders' contribution to IAS management (Figure 4). Nevertheless, some significant differences were detected. Especially outstanding were “nature users” (i.e. farmers, hunters), who significantly (Kruskal-Wallis: $F = 2.01$; $p < 0.001$) at-

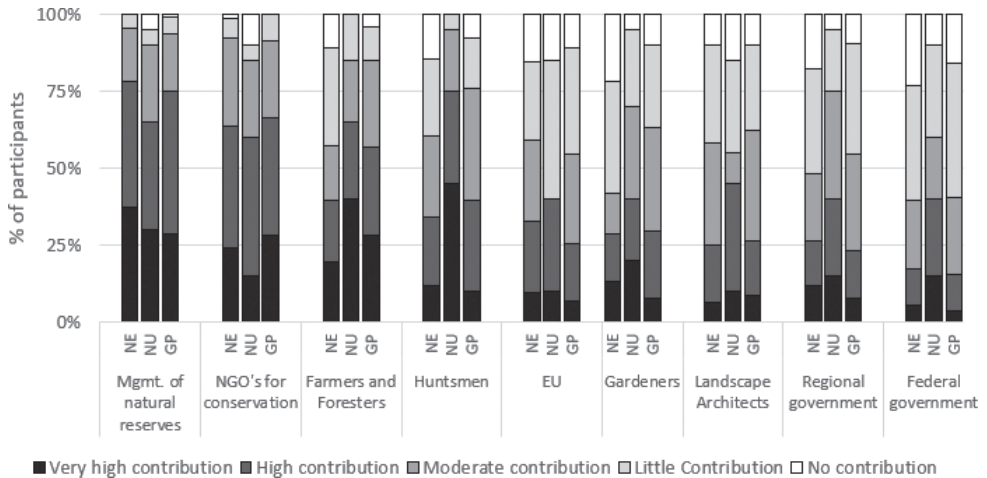


Figure 4. Assessment of the stakeholder groups opinion of the contribution to IAS management by different stakeholders. Differences were tested for significance by χ^2 -test and Fisher's exact tests – no differences detected. Significant differences between NU and NE were found in the “Farmers and Foresters” group (Kruskal-Wallis: $F = 2.01$; $p < 0.001$). Abbreviations: GU = general public; NU = nature users; NE = nature experts.

tested themselves a higher contribution to IAS management than nature experts did. Taken together, these results show that the participants assumed that dedicated institutions (e.g. NGO's and conservation area-managers) have the largest contribution to IAS management, while the contribution of political decision-makers is minor.

Assessment of the relevance of invasive alien species in Austria and EU policies

More than 75% of all participants replied that IAS and their management are 'a rather' to 'very relevant' topic for Austria and that it is "rather to very important" to manage them at EU level (Figure 5). However, more than 50% of the respondents of all stakeholder groups were not aware of the EU IAS Regulation. In particular, members of the general public were significantly less aware of this Regulation than the other two groups (X^2 -test: $F = 25.06$; $p = < 0.001$).

The results regarding the specific statements on EU IAS actions show that an overwhelming majority of participants agreed that IAS affect biodiversity in Europe (Figure 6). They disagreed the most with the statement that IAS were not a threat in EU countries. The majority of survey participants of all three stakeholder groups agreed that EU coordination in this field is advantageous and that coordinated activities for all EU member states were more efficient. Overall, the answers of the different stakeholder groups were quite similar, except for the answers of nature experts to the statement "every country should decide autonomously", where the agreement was significantly (X^2 -test: $F = 14.13$; $p = 0.007$) lower than for nature users.

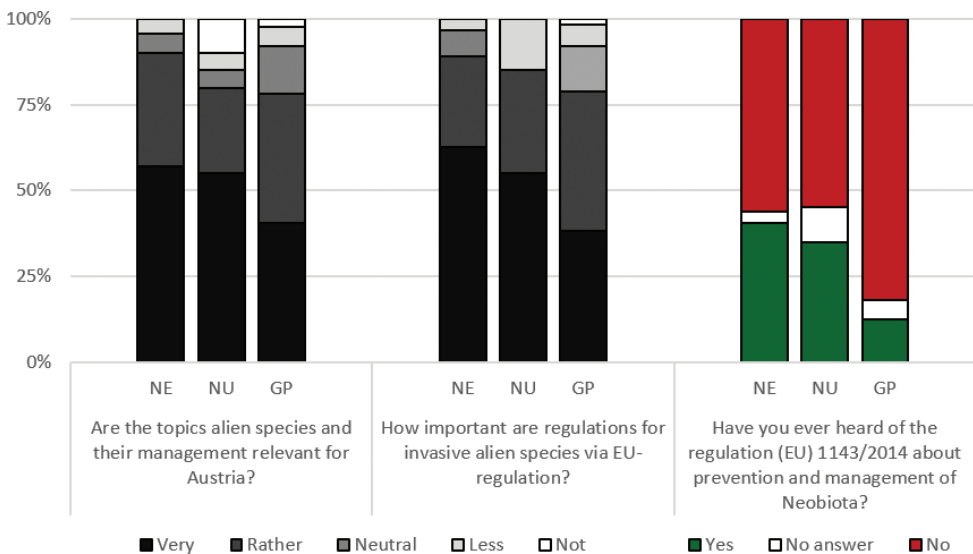


Figure 5. Relevance and awareness of the IAS topic and the EU regulation. Abbreviations: GP = general public; NU = nature users; NE = nature experts.

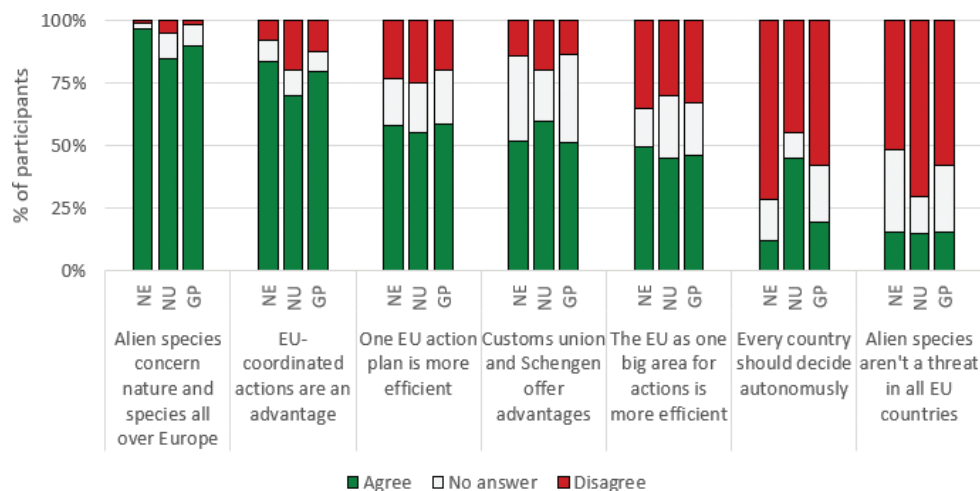


Figure 6. The importance of different IAS management and policy activities on the EU level for different stakeholder groups. Abbreviations: GP = general public; NU = nature users; NE = nature experts.

Discussion

Perception of species and acceptance of management methods

Since perceptions of IAS are diverse (García-Llorente et al. 2008) and some control methods can create highly emotional responses (Australian Academy of Science 2018; Bertolino and Genovesi 2003), it is necessary to include social perspectives into IAS research, management and policies (Kapitza et al. 2019). This study should encourage further research projects to raise mutual understanding for the views of the general public, nature users and conservation experts to achieve a broader consensus for IAS control measure.

The participants of this survey had a distinct knowledge about the origin of the species and the assessments of their ecological function and their belonging to Austrian ecosystems followed this pattern. Native species were more positively connoted than invasive alien ones across all three stakeholder groups. The physical appearance assessment showed that all species were rated as “aesthetic” (German: “optisch ansprechend bzw. schön”) or “very aesthetic” (German: “optisch sehr ansprechend bzw. sehr schön”) by the majority of the participants. In this context, we were particularly interested to test if there is a significant relationship between the aesthetic appearance of species and the acceptance of different management methods. Previous studies have shown that acceptability of management measures often reflect aesthetic motivations (Verbrugge et al. 2013; Fischer et al. 2014) but also that information about the impact of IAS lowers the aesthetic attraction and raises the acceptance of management measures (Junge et al. 2019). However, as the rating of the physical appearance of the study species was similar among the stakeholder groups, it was not possible to identify significant relationships with the assessment of management methods.

We found a significant correlation between the assessment of study species as belonging to Austrian ecosystems and the acceptance of lethal management. When a species was considered to be an invasive alien species, acceptance of lethal methods was significantly higher. This result confirms other studies that had found similar results for the acceptance of eradication measures for IAS with negative impacts on the environment (García-Llorente et al. 2008; Verbrugge et al. 2013; Fischer et al. 2014; Lewis et al. 2019). However, it is important to note that the acceptance of lethal management differs between stakeholder groups, especially for the mammal species of this study. Nature experts follow the described native/invasive alien-pattern, confirming previous studies which have shown that better knowledge of IAS increases the acceptance of control measures (Bremner and Park 2007). Further, nature users had a significantly higher acceptance of lethal methods than the general public. For example, this corresponds to the situation in New South Wales, where the general public opposed the killing of Brumbies (*Equus callabus*), while hunters and scientists supported it (NSW Government Office of Environment & Heritage 2016; NSW Threatened Species Scientific Committee 2016; Australian Academy of Science 2018; Parliament of New South Wales 2018). Similarly, Verbrugge et al. (2013) and Jaric et al. (2020) report that people are less likely to support eradication when it concerns a charismatic, aesthetically or otherwise attractive animal species, even if there is scientific evidence that it is invasive. Using the example of *Anser albifrons* and *Branta leucopsis* management on Islay (Scotland), Hanley et al. (2003) found that the willingness to pay for management measures was significantly reduced when lethal methods were included. In an expert survey on alien donkey control on Bonaire (Caribbean Netherlands), lethal methods were considered as least acceptable and fencing as most acceptable (Roberts et al. 2018). Estévez et al. (2015) stated that “value systems and risk perceptions are understood as the fundamental basis of discrepancies” among the different stakeholders. These value systems include aesthetic attraction and emotional bonding, as well as the utilisation of nature.

The overall rejection of chemicals (herbicides, poison pellets) as a method for killing invasive alien species was already shown in other studies (Verbrugge et al. 2013), where for mammals in particular, acceptance for this method is low. Nature users expressed a significantly higher acceptance of this method, but it was still low. Generally, nature users had higher acceptance levels for all management methods than the other two stakeholder groups. This may reflect the fact that members of this stakeholder group are economically directly dependent on extracting natural resources and thus negative impacts caused by IAS might be more evident for them. It has been shown that personal interests (e.g. economic interests) influence opinion held on specific alien species (Shackleton et al. 2019a, b). Further, García-Llorente et al. (2008) showed that conservation professionals and local citizens of the Donana region (SW Spain) considered effects of IAS to the local economy (while tourists considered the effects on threatened species) as economic incentives for IAS eradication. In a Swiss study, experts and members of the general public attribute a higher priority to ecological than to economic aspects (Junge et al. 2019). Further, utilisation can also be one value that influences the attitude of people towards nature (Estévez et al. 2015).

Since approximately 86% of the territory of Austria is used for agriculture or forestry (Statistik Austria 2016), nature users are the dominant social actor in environmental management. In our study, nature experts did not consider nature users as important for IAS management, although other studies have shown that nature users' knowledge and goals often do not differ fundamentally from their own (Badgley 2003). Badgley (2003) stated that "farmers can benefit from conservationists as advocates for farming practices that raise the quality of the landscape for farmers and for biodiversity and conservationists can benefit from farmers who enhance the ecological value of working landscapes for more native species". Therefore, we consider it crucial for nature users and nature experts to appraise each other's values and to work jointly to address problems caused by IAS.

In the disputed cases of failed grey squirrel eradication in Italy (Bertolino and Genovesi 2003) and Brumby eradication in Australia (NSW Threatened Species Scientific Committee 2016), animal right groups rejected lethal methods and established strong opposition to halt planned management measures. According to Crowley et al. (2017), conflicts concerning IAS management are not always avoidable, but taking the socioecological context into account, they can be minimised. For Perry and Perry (2008), the solution is communication and increasing understanding between "managers" (i.e. nature users, nature experts) and animal rights groups. Caravaggi et al. (2017) came to similar conclusions after surveying the opinions on lethal methods for IAS management of members and non-members of rural interest groups in Northern Ireland. Perry and Perry (2008) argue that managers should be more open to exploring non-lethal alternatives and animal rights groups should understand the motivation behind eradication attempts and be more involved in providing the extra funding necessary to support preventative measures and that "cooperation between the two groups is possible and desirable and that prevention of species invasion is an obvious area in which to begin." Our survey did not sample the opinions of animal rights activists, but it would be interesting to include them in a future study. Legal measures (as provided by Article 7 of the EU IAS Regulation 1143/2014) were received favourably by all three stakeholder groups. Our interpretation is that these measures are neither lethal for IAS nor do they affect the daily life of a significant proportion of participants, so ethical conflicts are likely perceived to be minor.

Across stakeholder groups, the participants' knowledge whether survey species were native or invasive alien species was very high. The majority of the participants assigned the species to the correct category. As the level of knowledge affects understanding and behaviour of people (Shackleton et al. 2019b), as well as perception of IAS (Eiswerth et al. 2011; Vaz et al. 2019) and control measures (Bremner and Park 2007; Junge et al. 2019), this may have had an influence on the present assessment of the perception parameters, as well as the management method acceptance parameters.

Relevance of EU IAS policies

Although the contribution of the EU to IAS management is rated low (nearly 50% responded that there is currently little or no contribution by the EU) among all stake-

holder groups, there is overwhelming support for more ambitious measures to be implemented at EU-level. The majority of the participants agreed with the advantages of IAS management organised and regulated by the EU. Thus, there is a high awareness of IAS and the survey participants are aware of the advantages of tackling this problem on a European level. For comparison, in a Swiss study only 40% of the participants belonging to the general public-stakeholder group were aware of the term invasive alien species (Junge et al. 2019).

Representativeness of this survey

This online survey used the convenience sampling method, i.e. the survey was open to everyone interested as long as (s)he lives in Austria. This approach is useful and widely used in cases when the basic sample size is unknown or very large, as is the case for the three stakeholder groups in this survey (Harvey et al. 2016; Lindemann-Matthies 2016). However, this approach comes with some limitations that have to be kept in mind when interpreting the results. First of all, it is unknown to what extent participants of the survey are fully representative for the respective stakeholder group, as biases, such as willingness to participate or basic knowledge of the existence of the survey, might be relevant (Etikan 2016). Secondly, sample sizes of the stakeholder groups differ substantially – as is the case in our survey with sample sizes varying between 20 (nature users) and 128 (general public). Finally, the substantial number of not-completed surveys may be associated with certain personal preferences which may also introduce specific biases in the results.

When distributing the survey, we used a broad set of communication channels for spreading the survey widely and thus reaching out to diverse audiences. In addition, the personal information of participants revealed that while some social strata (e.g. young urban populations) are somewhat over-represented, the distribution among basic demographic and personal parameters is relatively closely reflecting the Austrian population composition (Suppl. material 2: Table S1). Thus, we conclude that this survey provides important insights into the perception of native and invasive alien species in Austria. Still, it is clear that full representativeness cannot be achieved with convenience sampling.

Conclusions

Since the majority of the participants agreed that IAS concern Austria and that there is a need to regulate them on a European level, this study indicates substantial awareness of the topic. The high level of knowledge, whether it is a native or an invasive alien species, as well as the perception parameters in the survey, emphasise this finding. As other studies have shown, one key to success for raising the general public's awareness and support for IAS control measures is education and knowledge transfer (Bremner and Park 2007; Eiswerth et al. 2011; Junge et al. 2019). However, this can also cause

polarisation and trigger conflicts (Crowley et al. 2017) and therefore it has to be done wisely. An improved understanding of the acceptance of management methods among stakeholder groups is also crucial for avoiding future conflicts.

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Supplementary material 1

Text S1, S2

Authors: Raphael Höbart, Stefan Schindler, Franz Essl

Data type: text documents

Explanation note: **Text S1.** Original (German) version of the survey text. **Text S2.** Translated English version of the survey text.

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Supplementary material 2

Table S1. Overview on demographic data of survey respondents

Authors: Raphael Höbart, Stefan Schindler, Franz Essl

Data type: statistical data

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Trait–environment relationships of plant species at different stages of the introduction process

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Abstract

The success of alien plant species can be attributed to differences in functional traits compared to less successful aliens as well as to native species, and thus their adaptation to environmental conditions. Studies have shown that alien (especially invasive) plant species differ from native species in traits such as specific leaf area (SLA), height, seed size or flowering period, where invasive species showed significantly higher values for these traits. Different environmental conditions, though, may promote the success of native or alien species, leading to competitive exclusion due to dissimilarity in traits between the groups. However, native and alien species can also be similar, with environmental conditions selecting for the same set of traits across species. So far, the effect of traits on invasion success has been studied without considering environmental conditions. To understand this interaction we examined the trait–environment relationship within natives, and two groups of alien plant species differing in times of introduction (archaeophytes vs. neophytes). Further, we investigated the difference between non-invasive and invasive neophytes. We analyzed the relationship between functional traits of 1,300 plant species occurring in 1000 randomly selected grid-cells across Germany and across different climatic conditions and land-cover types. Our results show that temperature, precipitation, the proportion of natural habitats, as well as the number of land-cover patches and geological patches affect archaeophytes and neophytes differently, regarding their level of urbanity (in neophytes negative for all non-urban land covers) and self-pollination (mainly positive for archaeophytes). Similar patterns were observed between non-invasive and invasive neophytes, where additionally, SLA, storage organs and the beginning of flowering were strongly related to several environ-

mental factors. Native species did not express any strong relationship between traits and environment, possibly due to a high internal heterogeneity within this group of species. The relationship between trait and environment was more pronounced in neophytes compared to archaeophytes, and most pronounced in invasive plants. The alien species at different stages of the invasion process showed both similarities and differences in terms of the relationship between traits and the environment, showing that the success of introduced species is context-dependent.

Keywords

archaeophytes, functional traits, GABLIS, indigenous plants, introduced species, invasive species, native species, neophytes

Introduction

Invasive alien species (IAS) have a large ecological impact on the diversity and abundance of native plant species (Vilà et al. 2010; Pyšek et al. 2012). Worldwide, the number of naturalized vascular plant species reaches almost 14,000 with tendencies for further increase (van Kleunen et al. 2015, 2019; Pyšek et al. 2017; Seebens et al. 2019). Consequently, much effort has been invested to identify the main causes of invasiveness. Multiple studies have shown that certain functional plant traits promote invasiveness (e.g. Pyšek and Richardson 2007; Küster et al. 2008; van Kleunen et al. 2010; Tecco et al. 2010; Divíšek et al. 2018). However, a conclusive list of traits that promote successful invasion is lacking mainly due to ambiguous results that can, at least partly, be attributed to the context-dependence of the invasion process (Kueffer et al. 2013). Additionally, propagule pressure and introduction by humans (e.g. cultivation, ornamental purposes) have great effect on invasiveness (Pyšek et al. 2015).

The distribution of alien species is habitat-dependent (Chytrý et al. 2008a, Chytrý et al. 2008b; Dainese and Bragazza 2012), thus functional traits relevant in one type of habitat can become unimportant in another (Divíšek et al. 2018). Therefore, within one habitat the set of traits essential for survival and reproduction should be comparable across different groups of plants (native, non-invasive and invasive alien species). Alien species may thus share some traits with native species which can help them successfully establish under specific environmental conditions (Ordóñez et al. 2010), but which may differ among habitats. Yet, alien species exhibit certain dissimilarities to natives, which can be beneficial in the colonization of new environments and reduce competition (Pyšek and Richardson 2007; Divíšek et al. 2018). A meta-analysis by van Kleunen et al. (2010) confirmed that invasive species show significantly higher values for all trait categories analyzed (e.g. size, fitness, growth rate) than native and non-invasive species.

To perform comparative studies of the invasiveness of species, several approaches are possible, as conceptualized by van Kleunen et al. (2010). Of these, the most commonly performed are comparisons of (i) invasive with native species, and (ii) invasive with non-invasive species in the invaded area. However, such comparisons can be performed in both target (introduced) and source area (Parker et al. 2013).

Functional traits can be used to characterize the success of alien species over natives. Alien species ('exotic' or 'non-native' species) are classified, based on their residence time in the area, into 'archaeophytes' (alien species introduced before 1500 CE), and 'neophytes' (introduced after 1500 CE). Representatives of both groups can be classified according to the stage they reached in the invasion process into casual, naturalized or invasive; the latter term applies if they spread rapidly, become widely distributed (Richardson et al. 2000, Blackburn et al. 2011) and some have an impact on human well-being, ecosystem functioning, biodiversity, or human infrastructure (Vilà et al. 2010). Comparing traits of invasive and non-invasive plant species in their environmental context can help elucidate why some alien species become invasive, and others not (van Kleunen et al. 2010). Considering species at different stages of the invasion process can distinguish between traits that do not confer invasiveness (native vs non-invasive) from those that do (native vs invasive/potential invasive and invasive vs non-invasive) at each individual stage. The influence of traits on invasiveness can be challenging to interpret since it can differ depending on a species' residence time (how much time have alien species spent in their introduced area).

A range of environmental variables such as land cover, climate, and geological bedrock, have been shown to affect native and different groups of non-native species differently, and changes in land cover (providing a proxy for habitat) and/or climatic factors (particularly changes in temperature and rainfall amount and range) may particularly benefit invasive species (Hulme 2009). In Central Europe, both archaeophytes and neophytes prefer similar climatic conditions, reflecting their global environmental preferences, i.e. relatively warm and dry climate possibly due to their origin (Pyšek et al. 2005). Similarly, both groups of alien species are promoted by increasing land use intensity (Chytrý et al. 2008b, 2012; Polce et al. 2011). Accordingly, alien species can be favored when urban or agricultural land cover increases (neophytes and archaeophytes, respectively; Chytrý et al. 2008a). While geological heterogeneity strongly affects native species, this is not the case for archaeophytes, being mainly abundant on arable fields, i.e. homogenous land with fertile soils, while neophytes are strongly related to urban land cover (Kühn et al. 2003, 2004). Additionally, Tecco et al. (2010) showed that climate (temperature, precipitation, and frost), geological variables and land cover had a negative effect mainly on woody alien species and no significant effect on the herbaceous alien plants. Yet, the success of both native and alien species cannot be assessed in isolation from the environmental determinants of their distribution.

The reason why native and alien species may respond differently to environmental factors is often attributed to their functional traits. Wolf et al. (2020) showed a strong pattern of changes in functional composition with respect to environment in a rural–urban gradient. Traits relevant for the success of alien species are related to stress tolerance (i.e. SLA) or environmental disturbance (height, seed size) (Pyšek and Richardson 2008; Gallagher et al. 2015). Further, traits related to competitiveness (e.g. height) can prove beneficial for invasive species (Divíšek et al. 2018). For instance, phenology, in terms of earlier or longer flowering duration can be advantageous. Pyšek et al. (2003) showed that IAS might flower earlier or later than native species as a part of a "try harder" hypothesis. Pollination systems do not show significant links to invasion

success (Pyšek and Richardson 2008), but self-pollination tends to support the spread of neophytes more than any other type of pollination (Pyšek et al. 2011). However, the role of pollination in the invasion process is mainly studied without relation to environmental drivers. Kühn et al. (2006), though, showed that pollination types vary spatially and in relation to climatic, geological and land-cover factors.

Evaluating the relationships between the environment and plant functional traits is crucial for understanding the response of species of different origin and different stages in the invasion process to changing environmental conditions (climate change, land-cover change). Here, we quantified the relationships between climate, land cover and bedrock with relevant plant traits using a dataset with 1,300 plant species in Germany. We examined several groups of plants including natives and different subgroups of alien species across 1,000 randomly selected grid cells in Germany. The following questions are addressed: (i) Is there a relationship between plant traits and environment in native and alien species? (ii) How do these relationships depend on the residence time of plant species (with archaeophytes being introduced earlier and neophytes more recently)? (iii) How do these relationships differ between non-invasive and invasive neophytes?

Methods

Species occurrence

Species occurrence data was obtained from FLORKART (Federal Agency for Nature Conservation / Network Phytodiversity Germany; <http://www.floraweb.de>) for the period 1950–2013. FLORKART includes over 14 million records on species occurrences collected by thousands of volunteers. Species were analyzed at a spatial resolution of grid cells with 10' longitude \times 6' latitude (\sim on average 130 km² ranging from 117 to 140 km²). A presence/absence matrix was generated for a random sample of 1000 grid cells that contained at least 45 (out of 50) species that can be reasonably assumed to occur in every grid cell and serve as proxy for mapping quality (Kühn et al. 2006). This approach of grid cell selection ensured that chosen grid cells were properly surveyed. Additionally, some grid cells were smaller because they were located at the borders or along the coast. Thus, we excluded cells smaller than 117 km² (which is the size of the smallest grid cell that is not truncated by borders or coastlines). Individual matrices were generated for five groups of plants: native (976 species), archaeophytes (168 species) and neophytes (156 species), with 1,300 plant species in total; neophytes were further divided into (i) species featured in the German-Austrian Black List Information System of invasive species (GABLIS; Essl et al. 2011), with 26 species, and (ii) species not included in GABLIS, with 130 plant species. Following GABLIS (Essl et al. 2011), plants were classified into action black list (invasive with limited distribution) and management black list (invasive and widely distributed species). In our paper, we will refer to the species from GABLIS black list (action and management list) as invasive neophytes and to the ones that are not included in GABLIS as non-invasive neophytes.

Traits

Trait data for all plant species were obtained from the Database on Biological and Ecological Traits of the Flora of Germany, BiolFlor (Klotz et al. 2002; Kühn et al. 2004; <http://www2.ufz.de/biolflor/index.jsp>), and LEDA (Kleyer et al. 2008; <https://uol.de/en/landeco/research/leda/data-files>). These traits represent morphology, phenology and habitat preferences of all three groups of plant species: SLA, seed mass, height, storage organs, pollination vector, flowering period, urbanity and hemerobic level (Table 1).

Table 1. Functional traits, environmental associations (hemerobic level and urbanity) and invasiveness data (GABLIS list) and sources used for the analysis.

Trait	Values	Units/description	Source
Mean specific leaf area (SLA)	metric	mm ² /mg	LEDA
Seed mass	metric	mg	LEDA
Mean plant height	metric	m	LEDA
Storage organs	yes / no / multiple	Presence Absence Multiple storage organs	BiolFlor
Pollen vector	multiple / insect/ wind / self	Multiple pollination types Wind pollination Self-pollination (including two subgroups: selfing by a neighboring flower and selfing in an unopened flower) Insect pollination	BiolFlor
Flowering period	months	Beginning of flowering period End of flowering period Duration of flowering period	BiolFlor
Urbanity	1–5 values for different states of urbanity	1 – urbanophobic (species grows exclusively outside urban areas) 2 – moderately urbanophobic (species prefers non-urban areas) 3 – urbanoneutral (species has no preference), 4 – moderately urbanophilic (species grows predominantly in urban areas) 5 – urbanophilic (species grow exclusively in urban areas)	BiolFlor
Hemerobic level	level of naturalness with values 1–9	Polyhemerob and α -euhemerob, values 1–2 (species preference for artificial habitats) β -euhemerob and α -mesohemerob, values 3–4 (species prefers altered habitats) β -mesohemerob and α -oligohemerob, values 5–6 (species preference for moderately altered habitats); β -oligohemerob and γ -oligohemerob, values 7–8 (species prefers semi-natural habitats) Ahemerob, value of 9 (species preference for natural habitats)	BiolFlor
GABLIS sublist	no / ML / AL	Neophytes not present on the list Neophytes on the management Black list (ML) Neophytes on the action Black list (AL)	GABLIS

Environmental data

Climate data (temperature, precipitation; Table 2) were obtained from the ALARM project (Fronzek et al. 2012) for the period 1961–1990, land cover (Suppl. material 1: Table S2) data from the CORINE database (Bundesamt für Kartographie und Geodäsie, 2012), and geological data (Table 2) from a map of the German Federal Institute for Geosciences and Natural Resources (Bundesanstalt für Geowissenschaften und Rohstoffe, 1993), all scaled to the same resolution as the floristic maps.

Data analysis

We analyzed the relationship between traits and environment across native and alien plant species. For each group (natives, archaeophytes, neophytes, non-invasive and invasive neophytes) matrices of species presence/absence \times grid cell were created (S). Correspondingly, environmental matrices (environment \times grid cell, E) and trait matrices (traits \times species, T) for every status group were compiled. To directly associate matrices S with E and T , we used a fourth corner approach as implemented in the function *traitglm()* of *mvabund* in R (Warton et al. 2015). Fourth corner analysis combines S (first–upper-left–corner), E (second–lower-left–corner) and T (third–upper-right–corner). The fourth (missing–lower right) corner is generated as a matrix that describes the trait–environmental relationships. We checked for collinearity among environmental variables and excluded variables with $r > |0.7|$ (Dormann

Table 2. Environmental variables and their sources used in the 4th corner analyses of trait–environment relationships of plant species in Germany.

Variable	Abbreviation	Categories	Unit	Source
Temperature	tmn	- mean temperature of the coldest month	°C	Fronzek, Carter and Jylhä 2012
	tmx	- mean temperature of the warmest month		
Precipitation		- mean annual precipitation - precipitation range of the year	mm	Fronzek Carter and Jylhä 2012
Land cover	arable land (%)	Land cover proportion of: - arable land - natural and semi natural areas - urban areas	proportion	Corine Land Cover (CLC)
	natural cover (%)			
	urban cover (%)			
Number of CLC patches	CLC patches	Total number of land cover patches per grid cell		Corine Land Cover
Geological types		Proportion of subsoils: - calcareous - loess - sand	proportion	Bundesanstalt für Geowissenschaften und Rohstoffe
Number of geological patches	Geological patches	Total number of geological patches per grid cell (regardless of the number of geological types).		Bundesanstalt für Geowissenschaften und Rohstoffe

et al. 2013). The function *manyglm* presents a multivariate extension of GLM (generalized linear model) and calculates the coefficient estimates of GLMs fitted to all (explanatory) variables simultaneously (Wang et al. 2012). Coefficients describe how environmental predictors can be predicted by changes in traits. Further, we used the function *anova.traitglm()* based on bootstrapping with 99 permutations, to test for the statistical significance of trait–environment relationships in predicting presence of only non-native species (for computational reasons, see below) on all sites (Suppl. material 1: Table S1a–d). Since the response matrix *S* was binary multivariate data, we used binomial distribution.

The data analysis was performed using R, version 3.6.1 (R Core Team 2017). The analysis of a larger matrix (e.g. native species) took 19 days on a Dell PowerEdge R930 Server with 4 * CPU E7-8867 v4 2.4 GHz (72 Cores) and 6 TB RAM with Windows 2016.

Results

Overall, there was an increase in the number of prominent trait–environment relationships from native species to non-invasive archaeophytes, non-invasive and invasive neophytes (Fig. 1; Suppl. material 1: Tables S2a).

Native species

Native species in Germany showed high heterogeneity in their functional traits and habitat conditions; thus the relationships between traits and environment were weak (ranging from -0.0003 to 0.01; Suppl. material 1: Table S2a).

Archaeophytes

The frequency of archaeophytes well adapted to urban environmental conditions (urbanity; Fig. 1a; Suppl. material 1: Table S2b) increased with mean temperatures (of both warmest and coldest month), broader precipitation range, across natural and urban areas, and with the number of geological patches. Conversely, their frequency decreased with an increase in annual precipitation, the proportion of calcareous subsoil and total number of Corine Land Cover (CLC) patches per grid cell.

With higher temperatures of the warmest month, species with high seed mass, wind- or self-pollination, high level of naturalness and those beginning to flower early will increase, while those with a long flowering period will decrease. Increasing amounts of precipitation disadvantaged small species that prefer artificial habitats but promoted species with high SLA, seed mass, presence of storage organs and multiple storage, self-pollination, as well as early beginning and late end of flowering.

Neophytes

Mean annual precipitation and number of CLC patches showed a strong positive relationship with multiple storage organs, yet mean temperature of the coldest month negatively affected this trait (Fig. 1b; Suppl. material 1: Tables S2c). Both wind- and self-pollination were negatively influenced by mean annual precipitation, and wind pollination was positively related to temperature (of the coldest and warmest month), sandy substrates and number of geological patches. Increase in the temperature of the warmest month promoted urbanophilic species, while the temperature of the coldest month positively affected the duration and end of the flowering period. Mean annual precipitation showed a negative relationship with plant height, but positive effects on SLA and plants with multiple storage organs.

Non-invasive neophytes

Increasing winter temperature positively affected wind- and self-pollination and flowering duration, whereas tall urbanophilic species were negatively affected (Fig. 1c; Suppl. material 1: Tables S2d). Conversely, high summer temperatures were positively correlated with the frequency of tall urbanophilic non-invasive neophytes, and negatively with long flowering duration or larger SLA and seed size. An increase in the number of CLC patches favored insect-pollinated, urbanophilic plant species with higher SLA, while negatively affecting the abundance of long-flowering, self-pollinated species.

Invasive neophytes

The temperature of the warmest month was positively related to SLA, multiple storage organs, self-pollination and negatively to duration of flowering (Fig. 1d; Suppl. material 1: Tables S2e). In contrast, the temperature of the coldest month was negatively related to SLA and positively to hemeroby. Annual precipitation negatively affected the beginning of flowering, while the precipitation range was positively associated with SLA and self-pollination. The number of CLC patches had a positive relationship with multiple storage organs and a negative one with hemeroby.

Differences among invasive neophytes (black list) were positively associated with land cover and mostly negatively with geological predictors. Neophytes with a limited distribution in Germany (action list) had positive relationships with all three types of land cover and with number of CLC patches and negative associations with calcareous, sandy substrates and number of geological patches.

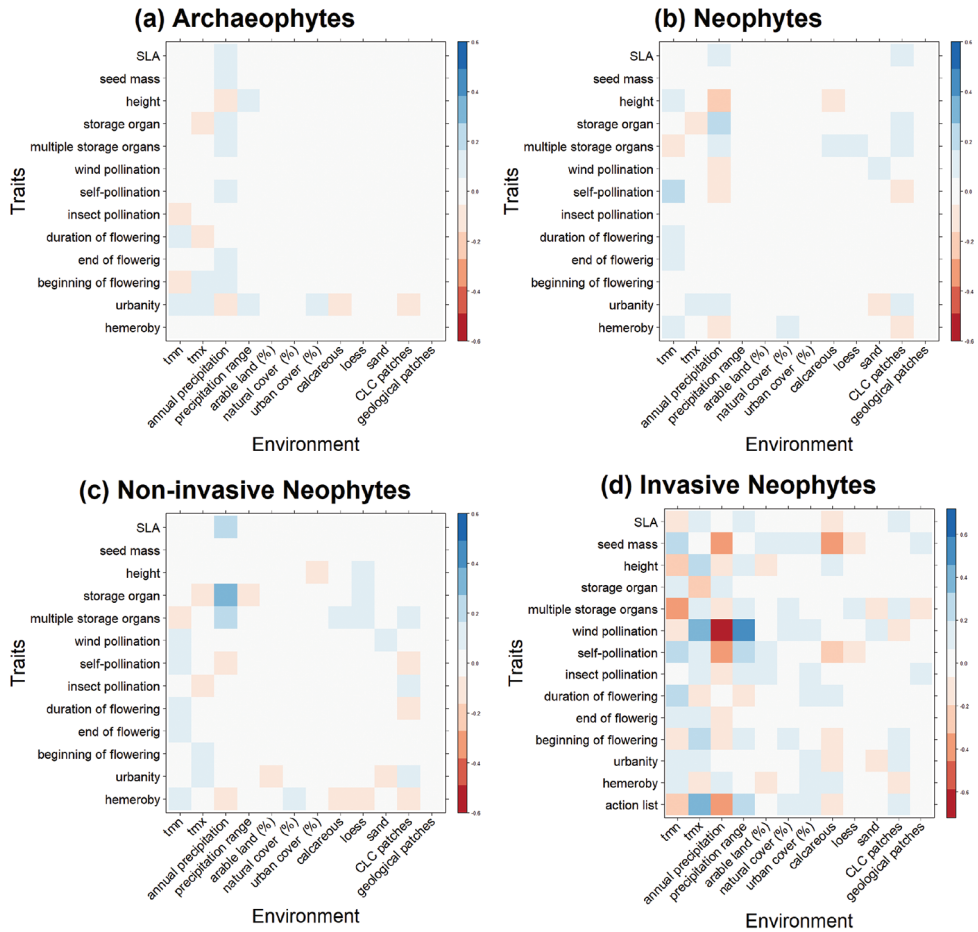


Figure 1. Fourth-corner plots for **a** archaeophytes **b** all neophytes lumped together regardless of status **c** non-invasive neophytes, and **d** invasive neophytes. Figure shows standardized interaction coefficients for plant traits (y-axis) and environmental variables (x-axis). Strong relationships are shown in blue (positive) and red (negative) while color intensity shows interaction strength with coefficient values on log scale. Abbreviations: tmn – mean temperature of the coldest month; tmx – mean temperature of the warmest month; CLC patches – total number of Corine Land Cover patches per grid cell.

Differences among groups

Archaeophytes and neophytes showed several contrasting trait–environment relationships (Fig. 1a, b). Specifically, the frequency of self-pollination in archaeophytes increased with the temperature of the warmest month, mean annual temperature and proportion of loess substrates, while under these conditions the frequency of neophytes diminished. Similarly, in archaeophytes we observed a positive relationship between

urbanity and temperature of the coldest month, the proportion of natural areas and number of geological patches, and a negative relationship with annual precipitation and number of land cover patches. Neophytes showed opposing trends.

Further, we observed differences between non-invasive neophytes and invasive neophytes (Fig. 1c, d). While the frequency of invasive neophytes with higher SLA increased with temperature of the warmest month and precipitation range, non-invasive neophytes displayed reversed trends. Similarly, urbanophilic invasive neophytes were promoted by increasing temperature of the coldest month, and insect-pollinated invasives by the number of geological patches and temperature of the warmest month, with contrasting tendency in non-invasive neophytes. Finally, insect-pollinated invasive neophytes benefited from increasing annual precipitation and a high number of land-cover patches, although these variables showed to be disadvantageous for non-invasive neophytes.

Discussion

We did not record any strong trait–environment relationships for native species, which may be due to the heterogeneity of different ecological groups. Preliminary tests (not shown) indicated that this scarcity of trait–environment relationships was not an artifact of the large sample size of native species. This is because (overall) native species colonize a much wider range of environmental conditions in their native range than species alien to that range. Alien species, for example, are rarely found under extreme environmental conditions such as in mountains, seashores, xeric habitats, bogs or fens (Chytrý et al. 2008, Alexander et al. 2011). This means that considering only alien plants makes it more likely to find trait–environment relationships than if all native species or random sets of native species (which are not ecologically selected) are taken into account. Therefore, we suggest analyzing trait–environment relationships of ecologically defined groups of native species. This, however, is beyond the scope of this paper; nevertheless, we report the results of native species to avoid publication bias.

We observed a lower number of strong trait–environment relationships for archaeophytes than neophytes, whereas in invasive neophytes (i.e. those on the GABLIS list) strong relationships were most frequent. Climatic variables had a high explanatory power in all groups. Traits of neophytes were mainly affected by climate and different geological types, and rarely by land cover. Most of the traits of archaeophytes were only affected by climatic conditions, such as temperature and mean annual precipitation (while precipitation range had little effect on their traits) and rarely by other environmental variables. Archaeophytes in Central Europe were predominantly introduced from the Mediterranean and the Middle East (Pyšek et al. 2012b), thus from a smaller range of geographical locations (and hence environmental conditions) than neophytes (introduced from around the globe), and many invasive neophytes originate from different regions and continents, and thus might have adapted to a wider range of environmental factors (Pyšek et al. 2005). This might be the reason why traits of neophytes, in addition to their diverse origin, showed a more pronounced response to climatic and geological factors.

We observed that relationships between environment and traits for different groups of alien species are more often similar rather than contrasting (e.g. height decreases with annual precipitation for both neophytes and archaeophytes; beginning of flowering shifts to earlier months with increasing winter temperature and precipitation for invasive and non-invasive neophytes, etc.). Plant growth (e.g. biomass, height, leaf size) and phenology are directly influenced by temperature (Hatfield and Prueger 2015); for example, extreme temperature (especially summer temperature) can alter the duration and success of the pollination process (Hegland et al. 2009). Furthermore, alien species exhibit traits that allow them to cope better than natives with the recently observed changes in climate or habitats, such as better dispersal ability, higher tolerance to climate change and higher competitiveness (Dukes and Mooney 1999).

Differences in neophytes vs. archaeophytes

As to the best of our knowledge, no statistical test allows the formal comparison of results across different fourth-corner analyses; we have to interpret differences among the trait-environment responses of different groups qualitatively. Trait-environment relationships were similar (positive or negative, respectively) for archaeophytes and neophytes in 13 cases but differed in seven cases. Primarily, urbanity expressed contrasting relationships, suggesting human-induced propagule pressure as an important driver. Neophytes tend to be more urbanophilic, thus the increase in temperature was positively related to this trait (urban heat island effect; Ricotta et al. 2009). Urban areas facilitate neophytes (Kühn et al. 2004; Kühn and Klotz 2006), and alien species are often associated with cities (Chytrý et al. 2008b; Knapp et al. 2009; Aronson et al. 2014). Some studies showed that neophytes are becoming a dominant group in urban areas (Chocholoušková and Pyšek 2003; Pyšek et al. 2004), while the association of archaeophytes with this type of environment decreased in recent decades, and they are more common in arable landscapes (Botham et al. 2009). Hence, the increase in the proportion of arable and natural land cover affected urbanophytic neophytes negatively, but the increase in the proportion of urban area increased their abundance (and resulted in a reversed trend in archaeophytes). Neophytes are cultivated in gardens and public parks (Reichard and White 2001; Pergl et al. 2016), and their spread is further facilitated by extensive transportation systems (Seebens et al. 2015). Consequently, cities often present harbors for the spread (von der Lippe and Kowarik 2008) and establishment of newly introduced species (Kühn et al. 2017).

The majority of neophytes (especially invasive) are pollinated either by insects or wind, whereas archaeophytes are often self-pollinated (Pyšek et al. 2011). Many agricultural weeds are self-pollinated archaeophytes, possibly due to a lack of suitable pollinators or because of abiotic stress. Further, in archaeophytes, self-pollination is more common with increases in the proportion of loess. This can be due to loess being very fertile and suitable for agriculture, so self-pollination can be an alternative (Kühn et al. 2006), especially with the increasing scarcity of insects in regions of intensive agriculture (Hallmann et al. 2017).

Differences in non-invasive vs. invasive neophytes

Flowering phenology is important for the successful spread of invasive species (Knapp and Kühn 2012). Plant species have evolved in tune to local climatic regimes in their native range or colonized such regions naturally. With increasing temperatures (summer and winter), invasive neophytes finish their flowering period later in the year (with overall shorter duration). However, higher summer temperatures had a negative effect on the duration and higher winter temperature caused invasive neophytes to start flowering earlier. Many invasive species in Germany originate from warmer climates and as a result, an increase in winter temperature can act as a switch to earlier flowering. Earlier flowering of invasive species compared to non-invasive may ensure their reproductive success, and higher summer temperatures prolong the flowering season to late summer (Knapp and Kühn 2012). Low precipitation often impedes flowering, and the species that flower earlier can avoid summer droughts (Godoy et al. 2009). The increase in precipitation range (usually resulting from wet winters and dry summers) decreases the duration of flowering and plants were flowering later in the year. Depending on the origin of invasive neophytes, we can expect different responses to current or future climatic conditions. Provided that climate in the introduced area is the same as in the native area, flowering phenology can stay the same. However, if introduced species are subjected to a different climate, the flowering depends on the capability of invasive species to adapt or respond plastically to new conditions.

Alien plants that have often been introduced for their aesthetic features as ornamental plants can attract pollinators (colorful and fragrant flowers) and divert them from native plants (Bjerknes et al. 2007; Muñoz and Cavieres 2008). The majority of tropical and temperate plants are insect-pollinated (Ollerton et al. 2011), invasive neophytes, though, are primarily insect or self-pollinated. Additionally, many invasive species are annual plants and when suitable pollinators are not available they are able to self-pollinate which can be beneficial for the successful invasion of new areas (van Kleunen et al. 2007).

Climatic factors did not have a different effect on the occurrence of invasive species from the management or action black list. Species on the action list are more likely to be found in all three types of land cover than those from the management list. We can, therefore, expect that species which are invasive but still of limited distribution, will spread, especially as habitats become more fragmented (occurrence of action list species shows an increase with CLC number of patches).

General patterns

Geological bedrocks did not have a major effect on most of the traits in different groups, despite explaining roughly a quarter of plant distribution variability in Germany (Pompe et al. 2008). Archaeophytes often occur on loess, which is highly productive and usually used for intense agriculture. However, in calcareous substrates ar-

chaeophytes tend to flower later while invasive neophytes flower earlier and are taller. Species-rich calcareous grasslands used to be common in Germany and are now frequently afforested, suffer from shrub encroachment or are surrounded by agricultural fields (Fischer et al. 1996). Sandy substrates can warm up earlier during winter and spring and can be suitable for neophytes introduced from warmer regions. Additionally, due to its low water-retention property, sandy substrates are frequently colonized by species adapted (i.e. having suitable traits) to drought.

Different land-cover types as well as the number of land-cover patches and geological patches had an effect on most of the traits of invasive neophytes, and very little (or no effect) on archaeophytes. Furthermore, landscape transformation and heterogeneity have an effect on invasive species in different stages of invasion and fragmentation of the landscape may facilitate the spread of invasive species (With 2002). Habitat heterogeneity intensifies invasion and increases dispersal (O'Reilly-Nugent et al. 2016; Dukes and Mooney 1999), and we have recorded a positive relationship with flowering phenology, SLA, height and seed mass of invasive neophytes. However, invasive neophytes with multiple pollination vectors (i.e. having different pollination types) benefited the most whereas wind-pollinated species colonized the least heterogeneous landscapes. These wind-pollinated invasive species are often dependent on specific habitats, for example, *Fraxinus pennsylvanica* or *Acer negundo* are often abundant in riparian or urban habitats (Burton et al. 2005).

Many studies have shown that functional traits of alien species are associated with invasiveness (Hamilton et al. 2005; Pyšek and Richardson 2007; Ordoñez et al. 2010; van Kleunen et al. 2010; Gallagher et al. 2015; Divíšek et al. 2018). However, the results were often ambiguous, possibly due to excluding environmental factors from analyses. In our study, we showed that traits, particularly of invasive neophytes, exhibit a strong relationship with the environment. Native species showed fewer associations with environmental factors as their traits may be more conservative in their native habitat and less likely to fluctuate. Yet, we looked at climatic conditions within a limited period (1961–1990) and native species might show significant changes in their functional traits as climate changes. Similar to native species, archaeophytes, the species that have settled in Germany for a long time, showed the least significant trait-environment relationships among alien species, while the traits of invasive neophytes are greatly affected by climate, geology and land cover. As discussed, this might be due to the fact that many invasive species were introduced from areas with different climatic or geological conditions and respond more flexibly to changes in the environment (Hellmann et al. 2008).

Invasive neophytes mainly show positive trait-environment relationships. Since the values for most of the traits increased with the incorporated environmental factors (especially climatic and land cover variables), we can expect future climate and land-cover change to affect invasive neophytes more strongly than other alien groups. We showed that climate may affect in particular SLA, insect pollination and phenology of invasive species, whereas land cover may mainly influence height, seed mass and wind pollination. Climate change could affect archaeophytes as well. They mainly showed

positive relationships with climatic variables, and their values increased with the increase in temperature and precipitation. Future studies on the relationship between functional traits and environment of invasive plants are required in order to examine the effects of climate change or land cover changes. There is evidence that climate change may promote invasiveness (Pyšek et al. 2005), thus distinguishing which traits of alien species are benefiting under different climatic scenarios, can be valuable for management implications.

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Supplementary material I

Tables S1, S2

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Data type: model output

Explanation note: **Table S1.** Coefficient values from *traitglm* model for (a) native species, (b) archaeophytes, (c) neophytes, (d) non-invasive neophytes, (e) invasive neophytes in Germany. Coefficients describe how traits are related to environmental conditions; values show strength of interaction and direction (positive/negative).

Table S2. Results of *anova.traitglm* for different groups of non-native species with 99 permutations (probability integral transform residual bootstrap (PIT-trap) block resampling which accounts for correlation in testing).

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Assessing the probability of freedom from pine wood nematode based on 19 years of surveys

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Abstract

Many quarantine pests, such as the pine wood nematode (PWN, *Bursaphelenchus xylophilus*), are surveyed annually in all EU countries. Although a lot of resources are spent in the surveys, the confidence in pest freedom achieved with them is not commonly analysed. We assessed the probability that Finland is free from PWN, based on the surveys done in 2000–2018. We used the methods employed in the risk-based estimate of system sensitivity tool (RiBESS), which has recently been recommended for quarantine pest applications. We considered two scenarios: 1) the surveys aimed to justify phytosanitary import requirements and to facilitate exports and 2) the surveys aimed to detect invasions early to enable eradication of outbreaks. These differed only in the pest prevalence that the surveys were expected to detect. The surveys appeared to support the assumption that PWN is not present in Finland, but they did not seem extensive enough to ensure early detection of invasions. The sensitivity of the import-export surveys was greater than 0.6 in 13 years, whereas that of the early detection surveys was always below 0.25. The probability of freedom achieved in 2018 following 19 years of surveys increased asymptotically with the mean time between invasions. For the import-export surveys, this probability was at least 0.95 unless the mean time between invasions was less than 13 years. For the early detection surveys, the probability of freedom was less than 0.73 unless the mean time between invasions was 63 years or more. The results were rather robust with respect to the parameters for which exact information was lacking. To improve the assessment, a quantitative estimate of the probability of PWN invasion to Finland and a thorough assessment of the maximum area of an eradicable infestation would be needed. To gain an understanding about the true impact of quarantine pest surveys on biosecurity, more assessments, like the one presented in this paper, are needed.

Keywords

design prevalence, European Union, legislation, plant health law, quarantine pest, sensitivity

Introduction

All countries of the European Union (EU) are required, by legislation, to conduct annual surveys for several quarantine pests, such as the pine wood nematode (PWN, *Bursaphelenchus xylophilus*) (European Council 2000; EU 2016). One aim of the surveys is to show pest freedom to justify phytosanitary import requirements and to facilitate export to countries with corresponding requirements. In addition, the hope is that the surveys will detect pest invasions early enough to enable successful eradication of outbreaks. However, the confidence in pest freedom achieved with the surveys is not commonly assessed and thus their impact on biosecurity is not known.

PWN is the causal agent of pine wilt disease, which, under suitable conditions, can lead to mass mortality of susceptible pine trees (e.g. Futai 2013). It is thought to be native to North America and has been introduced in Asia, in Japan, China, Taiwan and South Korea (Mamiya 1988; Tzean 1997; Shin 2008; Zhao 2008) and in Europe, in Portugal and Spain (Mota et al. 1999; Robertson et al. 2011).

PWN can spread over long distances through the transport of wood and wood packaging material (Evans et al. 1996; EPPO 2009). From tree to tree, it is spread by longhorn beetles of the genus *Monochamus* via feeding and oviposition (e.g. Linit 1988). Feeding by an infested vector transmits PWN to healthy trees, whereas when being spread via oviposition, PWN is transmitted only to weakened trees, recently felled logs or logging waste, as the vectors do not breed on healthy trees (e.g. Akbulut and Stamps 2012). The most susceptible hosts to PWN are in the genus *Pinus*, but other conifers such as *Abies*, *Picea* and *Larix* can also be attacked (e.g. Takeuchi 2008).

PWN is not expected to cause pine wilt disease in areas where the mean temperature of the summer months is below 20 °C (Evans et al. 2008; Gruffudd et al. 2016). Hence, in much of Northern Europe, including Finland, PWN is unlikely to cause any symptoms. In such conditions, PWN is very unlikely to spread further from trees infected by feeding of the beetles. Moreover, as visible symptoms are not expected in these areas, PWN surveys must be based solely on laboratory analysis of asymptomatic samples.

In the EU, PWN is a quarantine pest, whose introduction into and spread within the Union is prohibited (European Council 2000; EU 2016; European Commission 2019a). Moreover, after PWN was first detected in the EU in 1999 (Mota et al. 1999), specific emergency measures that aim to prevent its further spread have been in force (EU 2012). The measures require all EU countries to conduct annual surveys to determine whether PWN is present in their territory.

In addition to PWN, EU member states must carry out annual surveys for several other quarantine pests. Regular surveys must be carried out for all quarantine pests and the so-called priority pests, such as PWN, must be surveyed every year (EU 2016; European Commission 2019b). The surveys of the priority pests must include a sufficiently high number of visual examinations, sampling and testing to ensure, as far as possible, the timely detection of the pest, with a high degree of confidence.

Due to these requirements, a lot of resources are being used in surveys of quarantine pests in the EU. For example, in the PWN surveys, approximately 16,000–21,000 samples were collected and analysed annually in 2014–2016 (European Commission

2018). In Finland alone, the cost of the PWN survey in 2000–2018 was up to approximately 100,000 euros per year (unpublished estimate based on information obtained from the Finnish Food Authority). Despite such significant investments, we did not find any published assessments of the confidence in pest freedom achieved with the surveys. However, if the confidence were assessed, it could be used to evaluate the benefit of the surveys and possibly to cut down the resources needed for future surveys.

The European Food Safety Authority (EFSA) is currently training the national plant protection organisations (NPPOs) of EU countries to plan the surveys required by the EU legislation with the risk-based estimate of system sensitivity tool (RiBESS) (EFSA 2012; EFSA 2018). The tool is based on principles presented by Cannon (2002) and Martin et al. (2007) and it was originally designed for estimating the sample size needed in the surveys of *Echinococcus multilocularis* infections in dogs and for calculating the survey sensitivity once the samples are collected (EFSA 2012). The methods employed in the tool have been used for designing surveillance of invasive species, including plant pests (e.g. Dominiak et al. 2011; Kean et al. 2015). However, quantitative assessments of the confidence in pest freedom are still exceptions rather than the rule.

We used the methods employed in RiBESS to assess the sensitivity of the annual PWN surveys carried out in Finland in 2000–2018 and the probability that Finland was free from PWN in 2018. We made these assessments for two separate scenarios with different assumptions: a) while assuming that the surveys were done to justify import requirements related to PWN and to facilitate exports to countries with respective requirements and b) while assuming that the surveys were aimed to detect invasions at an early stage to facilitate eradication. We show what kind of information is needed in the analysis and how the uncertainties of that information can be accounted for. Additionally, we highlight the value of quantitative estimates of the probability of pest invasion and demonstrate the dangers of using a seemingly uninformative prior probability of pest freedom when accumulating evidence for pest freedom from multi-annual surveys.

Methods

The surveys

PWN surveys were conducted in 2000–2018 in all the fifteen Centres for Economic Development, Transport and the Environment of Finland (Fig. 1), but the self-governing province of the Åland Islands was not included in the surveys. The survey was conducted by inspectors of NPPO of Finland and regional bodies to which the tasks had been delegated.

The main body of the surveys consisted of sampling of wood of PWN host plants, i.e. Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*). However, in 2012–2018, the PWN vector beetles present in Finland (*M. galloprovincialis*, *M. sutor* and *M. urussovii*) were also sampled using pheromone traps.

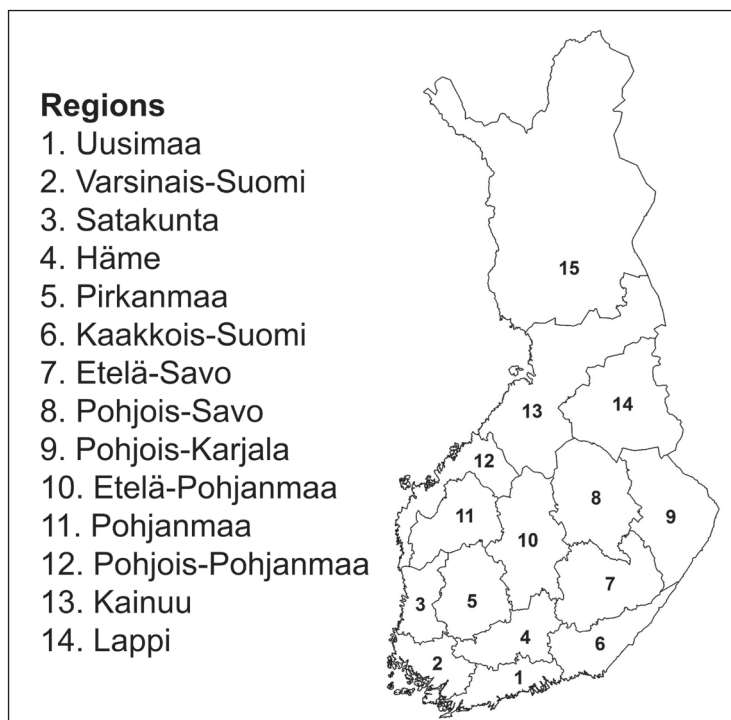


Figure 1. The fifteen administrative regions covered in the survey.

Wood sampling

Wood sampling was done according to the PWN survey guidelines of the NPPO, which were based on the EU PWN survey protocol (European Commission 2009). Samples were taken from risk areas, i.e. areas where the likelihood of PWN introduction is elevated and from regular forest areas. The risk areas were defined as pine forests at 5 km radius from harbours, industrial areas, landfills, wood storage areas and locations that receive imported wood packaging material.

All samples were taken from trees, wood or logging residuals that had signs of *Monochamus* activity or from pine trees that were dead or dying for no apparent reason. Each sample contained 0.5 l of wood chips and it was collected from an area that was, at most, 2 ha. If the whole sample was taken from one tree or a pile of logs, the distance between two samples was at least 200 m.

Samples were taken and stored so that their temperature was held below 26 °C, to ensure that the nematodes did not die in the process, as they needed to be alive to be detected. All samples were collected between April and October to maximise the probability that adult nematodes also would be present in the sample, as only adults can be identified to species, based on morphological features.

The number of wood samples collected from the different administrative regions in 2000–2018 is presented in Table 1.

Table 1. The number of wood samples collected in 2000–2018.

Region	Number of wood samples																		
	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018
Uusimaa	100	4	5	37	57	61	65	55	52	45	45	45	49	47	45	20	24	30	13
Varsinais-Suomi	75	3	3	30	50	50	51	29	45	50	45	57	45	45	46	46	46	45	20
Satakunta	75	0	0	50	50	50	40	46	50	50	42	43	47	43	45	45	45	42	45
Häme	50	0	0	0	0	0	0	25	25	25	19	18	22	19	20	20	20	22	21
Pirkanmaa	50	0	0	0	0	0	0	25	25	25	20	20	19	21	20	5	20	20	20
Kaakkois-Suomi	100	8	0	72	98	95	27	23	33	40	36	40	22	29	41	22	30	31	33
Etelä-Savo	50	1	0	0	0	0	0	54	52	53	46	46	45	45	45	45	45	50	23
Pohjois-Savo	50	0	0	0	0	0	0	24	23	26	28	20	17	10	20	10	20	7	29
Pohjois-Karjala	75	0	0	50	50	38	52	55	23	38	67	33	29	39	47	16	22	19	22
Keski-Suomi	50	0	0	0	0	16	52	52	53	51	45	47	29	9	20	20	15	20	20
Etelä-Pohjanmaa	50	0	6	0	0	0	0	25	25	27	21	20	20	20	20	20	20	20	20
Pohjanmaa	75	0	0	0	48	0	0	50	50	50	45	45	45	44	45	45	45	45	45
Pohjois-Pohjanmaa	75	0	0	51	54	51	50	54	50	53	45	45	45	44	45	45	20	17	16
Kainuu	50	0	0	0	0	0	16	28	15	27	20	20	19	28	20	9	9	9	5
Lappi	50	0	0	4	0	0	0	0	13	23	23	5	15	20	16	20	20	20	20
Total	975	16	14	294	407	361	353	545	534	583	547	504	468	463	495	388	401	397	352

Monochamus trapping

The traps were placed in places that were attractive to *Monochamus* beetles, such as storage areas of wood with bark and places with plenty of fresh logging residuals. The distance between traps was at least 500 m. The traps were set up in early June, inspected every other week and taken down at the end of August.

The trap type and attractant used varied between years and locations. Both multi-funnel and cross-vein traps and several pheromone and kairomone products, such as Gallowit, Galloprotect 2 D, and Galloprotect Pack, were used. In addition, some beetles were collected by hand. All samples were mailed to the laboratory with an ice brick that kept them cool.

The number of traps and the number of *Monochamus* individuals caught in the different administrative regions in 2012–2018 are presented in Table 2.

Table 2. The number of traps used and the number of *Monochamus* individuals captured in 2012–2018. In some of the regions and years, *Monochamus* were caught by hand and, therefore, the number of *Monochamus* can be positive even though the number of traps is zero.

Region	Number of traps / <i>Monochamus</i>					
	2012	2013	2014	2016	2017	2018
Uusimaa					2 / 1	2 / 0
Kaakkois-Suomi	6 / 0	6 / 5	6 / 9	6 / 0	0 / 6	0 / 1
Etelä-Savo					0 / 2	
Pohjois-Savo					0 / 1	
Pohjois-Karjala	2 / 0	2 / 0	2 / 0	2 / 0		
Pohjois-Pohjanmaa						0 / 4
Kainuu	2 / 0	2 / 0	2 / 18	2 / 0		
Total	10 / 0	10 / 5	10 / 27	10 / 0	2 / 10	2 / 5

Analysis of the samples

Extraction and identification of nematodes from the samples was done by the authorised plant health laboratory of Finland, according to a protocol that was based on the standards of the European and Mediterranean Plant Protection Organization (EPPO) on nematode extraction (EPPO 2013a) and on the diagnostics of PWN (EPPO 2013b) and on Hooper (1986) and Bergdahl et al. (1991).

Wood samples were first incubated at 20–25 °C for 14 days to allow the nematodes to reproduce. Then, the nematodes were extracted to a Petri dish using the Baermann funnel technique (Baermann 1917).

From the *Monochamus* samples, nematodes were extracted by sectioning the beetles to four parts and by leaving them on a Petri dish with water overnight. From the Petri dish, nematodes were searched using a stereomicroscope. If potential PWN were found, they were placed on pine discs to moult to adults and to reproduce. After the discs had been incubated at 20–25 °C for 14 days, the nematodes were extracted to a Petri dish using the Baermann funnel technique (Baermann 1917).

While in the Petri dish, the adult nematodes were searched under a stereomicroscope and all potential PWN were placed on a microscope slide for morphological identification. From 2011 onwards, if PWN had been found, the identification would have been verified using a Real-time PCR protocol (François et al. 2007; Ye 2012).

Entry sites, risk areas, and the target population

The survey guidelines were based on the assumption that the probability of PWN introduction was elevated in harbours, industrial areas and landfills and that the probability of PWN infestation was elevated at 5 km radius from such areas. In this paper, the areas with elevated probability of PWN introduction (i.e. harbours, industrial areas and landfills) are referred to as entry sites. The areas with PWN host plants at 5 km radius from entry sites are referred to as risk areas.

In principle, the survey design was risk based, since samples were collected from the risk areas and from regular forest areas and the sampling site type was recorded for each sample. However, when we delineated the spatial extent of the risk areas using the Finnish Corine Land Cover 2012 data at 20-m² resolution (Härmä et al. 2015), we found that, in thirteen of the fifteen administrative regions, the risk areas covered more than 80% of the area with PWN host plants (see Table 3). Since the inspectors did not have such delineation available when collecting the samples, we considered that the sampling site type data were likely to be flawed and decided not to use it. As sampling in the remote locations that did not fit the definition of the risk areas was probably rare, we assumed that all sampling was done in the risk areas. Thus, the target population of the survey was the risk areas.

Table 3. The area with PWN host plants, the area of entry sites (*EA*), the area of the target population (*Pop*, i.e. risk areas) and the mean area covered with PWN host plants in hypothetical PWN infestations with a 20-km diameter (*InfA*). Entry sites are areas with elevated probability of PWN introduction, i.e. harbours, industrial areas and landfills. Target population is the areas with PWN host plants at 5 km radius from entry sites.

Region	Host plants, km ²	Entry sites, km ²	Target population, km ²	Host plant area in hypothetical infestations, km ²
Uusimaa	5,640	940	5,260	156
Varsinais-Suomi	6,713	604	5,678	149
Satakunta	5,609	474	5,486	185
Häme	7,285	486	7,199	192
Pirkanmaa	9,566	546	9,475	202
Kaakkois-Suomi	8,042	604	7,774	170
Etelä-Savo	11,832	214	11,102	202
Pohjois-Savo	13,593	454	12,657	209
Pohjois-Karjala	15,003	293	12,301	200
Keski-Suomi	14,220	384	13,681	224
Etelä-Pohjanmaa	9,520	380	9,423	210
Pohjanmaa	9,391	443	8,437	187
Pohjois-Pohjanmaa	27,607	667	22,717	212
Kainuu	19,150	209	13,627	239
Lappi	61,783	538	31,229	210
Total	224,956	7,236	176,046	

Defining the aim of the survey with design prevalence

Proving that a pest is absent from a host population is not possible unless all members of the population are tested with a perfect test. Therefore, the aim of a survey must be defined in terms of design prevalence and sensitivity. Roughly, design prevalence determines the minimum prevalence that the survey is aimed to detect and sensitivity determines the probability with which the survey is expected to succeed in this aim. If the pest prevalence is equal to or greater than the design prevalence, at least one infested individual will be detected in the survey, with the probability equal to the sensitivity of the survey.

If the survey has not yet been done, the number of samples needed can be determined so that the survey fulfils its aim and proves that the pest prevalence is below the design prevalence with the desired sensitivity. In an ex-post analysis, such as this study, the sensitivity of the surveys, given a predefined design prevalence, can be determined, based on the number of samples taken.

Since the aim of the Finnish PWN surveys was not predefined in terms of design prevalence, we had to start by doing that. We did it by assuming the aim was a) to provide evidence to justify import requirements related to PWN and to facilitate export to countries with corresponding requirements and b) to detect possible PWN invasions early enough to enable successful eradication. These two cases were analysed separately. The first is referred to as the import-export survey and the latter, as the early detection survey.

The sensitivity of the surveys was assessed so that each wood sample and *Monochamus* trap was assumed to represent an inspection of a fixed-sized area with PWN host plants, i.e. the inspection site. Therefore, design prevalence had to be defined at two levels, namely, at the level of inspection sites (local-level design prevalence) and at the level of the administrative regions and Finland (regional- and/or national-level design prevalence).

The local level design prevalence refers to the proportion of PWN-infested wood objects and *Monochamus* beetles per inspection site. Regional- and/or national-level design prevalence refers to the proportion of PWN-infested area (where the PWN prevalence is at or above the local level design prevalence) of the total target population (i.e. risk area) in the region or the country.

At both levels, design prevalence had to be such that PWN could reach it, at least at some point in time, if it were established in the considered area. Additionally, design prevalence had to be such that it corresponded to, at least, one whole infested unit (i.e. wood object, *Monochamus* beetle or inspection site) per considered area (i.e. inspection site, region or country). The design prevalences used in this study are summarised in Table 4 and the justification for them is given below.

Local level design prevalence

We defined the local level design prevalence, based on the prevalence of *Bursaphelenchus mucronatus* in the wood samples collected in the Finnish PWN surveys in 2012–2018. This was considered appropriate, as *B. mucronatus* is closely related to PWN, widely established in Finland in coniferous forests (Tomminen et al. 1989) and, like PWN, it is vectored by *Monochamus* beetles (Tomminen 1990). Furthermore, *B. mucronatus*

Table 4. Local-, regional- and national-level design prevalences and effective probabilities of infestation used in the import-export and early detection surveys.

Parameter	Import-export	Early detection
Local-level design prevalence for the wood sampling component of the survey (DP_{wood})	0.12	0.06
Local-level design prevalence for the <i>Monochamus</i> trapping component of the survey ($DP_{Monochamus}$)	0.09	0.045
National-level design prevalence (DP_n)	0.01	
Effective probabilities of infestation for the import-export survey (EPI) and regional-level design prevalence for the early detection survey (DP_r)		
Uusimaa	0.020	0.030
Varsinais-Suomi	0.013	0.026
Satakunta	0.010	0.034
Häme	0.010	0.027
Pirkanmaa	0.012	0.021
Kaakkois-Suomi	0.013	0.022
Etelä-Savo	0.005	0.018
Pohjois-Savo	0.010	0.017
Pohjois-Karjala	0.006	0.016
Keski-Suomi	0.008	0.016
Etelä-Pohjanmaa	0.008	0.022
Pohjanmaa	0.009	0.022
Pohjois-Pohjanmaa	0.014	0.009
Kainuu	0.004	0.018
Lappi	0.011	0.007

does not cause any symptoms (Tomminen 1993), which is expected to be true also for PWN in the current Finnish climate (Gruffudd et al. 2016).

Information about the presence or absence of *B. mucronatus* was available for 2,876 wood samples and *B. mucronatus* was detected in 353 of these samples. Thus, using the binomial probability distribution, the apparent prevalence of *B. mucronatus* in the wood objects that were considered suitable for sampling in the PWN survey was estimated to be 0.12, with 95% confidence limits of 0.11 and 0.14.

To translate this estimated apparent prevalence to true prevalence, the sensitivity of the analysis (i.e. the probability that the pest is detected in the analysis, given that it was present in the object from which the sample was taken) should be known. Unfortunately, this information was not available for *B. mucronatus* or PWN. However, we concluded that by defining the local level design prevalence as the apparent local level design prevalence, we could link it directly to the estimated apparent prevalence of *B. mucronatus*. This is because the sensitivity of the analysis is likely to be roughly similar for the two species and, thus, a given true prevalence is likely to result in a similar apparent prevalence for both species.

To define the local level design prevalence for the *Monochamus* trapping component of the survey so that it matched the local level design prevalence of the wood sampling component, we used an estimate provided by Økland et al. (2010). They estimated that most likely 75% of *Monochamus* offspring emerging from PWN-infested objects are infested with PWN. Based on this, we assumed that the PWN prevalence in *Monochamus* adults should be 75% of that in wood objects suitable for sampling, i.e. 0.09. This is a rough estimate since it is based on the apparent prevalence of *B. mucronatus* in the wood samples instead of the true prevalence of *B. mucronatus* in wood objects, used for breeding by *Monochamus* beetles.

Finally, for the import-export surveys, the apparent local level design prevalence was set equal to the estimated apparent prevalence of *B. mucronatus* (Table 4). This was assumed to represent a prevalence of a PWN population that has been established long enough to reach its maximum density. For the early detection surveys, the apparent local level design prevalence was set to half of the estimated apparent prevalence of *B. mucronatus* (Table 4). This was assumed to represent a prevalence of a PWN population that is in the exponential phase of the sigmoid growth curve, i.e. the population is established, but still clearly growing.

Regional- and/or national-level design prevalence

When the aim of the survey is to show pest freedom to justify import requirements or to facilitate exports, design prevalence at the level of the region or country cannot be defined objectively. Furthermore, the design prevalence that should be used in such surveys is not defined in the Food and Agriculture Organization of the United Nations (FAO) standard on surveillance (FAO 2018) or the one on the requirements for the establishment of pest-free areas (FAO 2017). In this study, the national-level design prevalence of the import-export survey was set to 0.01, corresponding to 1,760 km² of forest with coniferous trees.

The probability that the region j is infested, given that the country is infested at the national-level design prevalence, was defined by the effective probability of infestation (EPI). It was calculated for each region as (Martin et al. 2007; Efsa 2012)

$$EPI_j = DPn \cdot \frac{RP_j}{\sum_{j=1}^{15} (PropPop_j \cdot RP_j)} \quad (1)$$

where j denotes the administrative region, DPn = the national-level design prevalence, RP_j = the relative probability of PWN invasion to the region j and $PropPop_j$ = the proportion of the target population (i.e. risk areas) in region j of the target population in Finland. The relative probability of PWN invasion to region j (RP_j) was assumed to be equal to the proportion of the area of entry sites in region j of the area of entry sites in Finland, i.e.

$$RP_j = \frac{EA_j}{\sum_{j=1}^{15} EA_j} \quad (2)$$

where EA_j = the area of entry sites in region j . The area of entry sites was obtained from the Finnish Corine Land Cover 2012 data with a resolution of 20 m² (Härmä et al. 2015) (Table 3). The effective probabilities of infestation defined in the above manner for the import-export survey ranged from 0.004 to 0.02 (Table 4).

For a survey that aims to detect invasions early enough to enable the eradication of outbreaks, regional- and/or national-level design prevalence can be determined, for example, based on the maximum area from which eradication could be attempted. The EU emergency measures for PWN (EU 2012) allow member states to refrain from attempting eradication if the diameter of the infested area exceeds 20 km. Therefore, we assumed that, in the early detection survey, infestations should be detected before they reach this size.

The regional-level design prevalences (DPr) of the early detection survey were defined assuming that, within the early stages of invasion, PWN infestations would be confined to one region. Thus, the regional-level design prevalences were calculated as:

$$DPr_j = \frac{InfA_j}{Pop_j} \quad (3)$$

where $InfA_j$ = the mean area covered with PWN host plants in hypothetical PWN infestations with a 20-km diameter in region j and Pop_j = the area of the target population (i.e. risk areas) in region j .

To estimate the mean area with PWN host plants in hypothetical PWN infestations with a 20-km diameter ($InfA_j$), we assumed that the infested area would be circular and that its centre would be in an entry site. Then to simulate such circular PWN infestations with a 20-km diameter, we selected hundred points randomly in the entry sites of each administrative region and delineated the area at 10-km radius from the randomised points. Finally, we calculated the mean area with PWN host plants within those areas separately for each region. The regional-level design prevalences defined in the above manner for the early detection survey ranged from 0.007 to 0.034 (Table 4).

Assessment of the probability of freedom from PWN

We assessed the probability of freedom from PWN with the methods used in RiBESS (EFSA 2012), which is based on principles developed by Cannon (2002) and Martin et al. (2007). We applied a hierarchical procedure 1) starting from the sensitivity of inspections per inspection site, 2) moving on to the sensitivity of the annual surveys at the regional and 3) the national level and 4) finally arriving at the probability of freedom achieved, based on the multiannual survey at the regional and national level. The hierarchy of the calculation of the sensitivity of the annual surveys is presented in Figure 2.

For some of the parameters needed in the assessment (such as the density of wood objects suitable for sampling and the density of *Monochamus* adults), information was uncertain or lacking. To account for this, the parameters were expressed as probability distributions and the assessment was done with Monte Carlo simulation. The number of iterations used was 10,000 in all the simulations. The simulations were done with R version 3.52 (R Core Team 2018) and the package mc2d version 0.1–18 (Pouillot and Delignette-Muller 2010).

The sensitivity of inspections

The sensitivity of inspections (inspection sensitivity, ISe) is the probability that the pest will be detected at an inspection site when it is present in the site at a prevalence equal to the local-level design prevalence. Inspection sensitivity was assessed separately for wood sampling (ISe_{wood}) and *Monochamus* trapping ($ISe_{Monochamus}$). It was calculated, based on the hypergeometric probability distribution, which is suitable for assessing the sensitivity of sampling from a finite population. The round of inspection sensitivity for hypergeometric distribution is (Cameron and Baldock 1998):

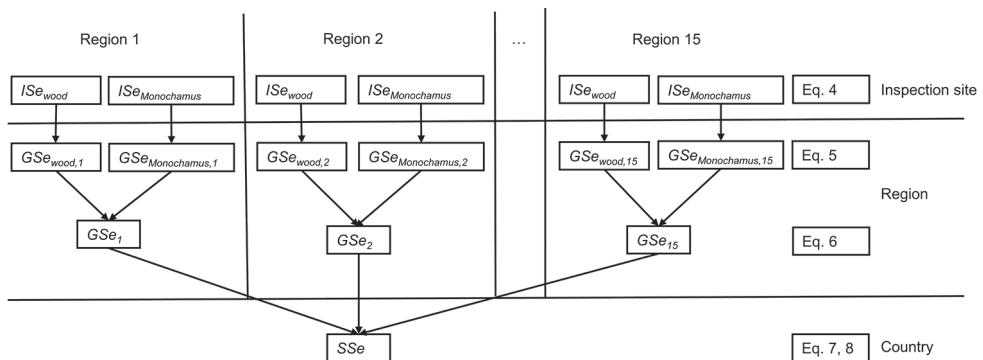


Figure 2. A schematic presentation of the analysis of the sensitivity of the annual surveys. The equation numbers (Eq. 4–8) refer to the equations presented in the main text. Abbreviations: ISe = the sensitivity of the inspections, GSe = the sensitivity of the annual surveys in the fifteen administrative regions and SSe = the sensitivity of the annual surveys in Finland.

$$ISe_i \cong 1 - \left(1 - \frac{n_i \cdot TSe_i}{p_i - 0.5 \cdot (p_i \cdot DP_i \cdot TSe_i - 1)}\right)^{p_i \cdot DP_i} \quad (4)$$

where i denotes either the wood or *Monochamus* and p_i = the total population size, i.e. the number of wood objects suitable for sampling or the number of adult *Monochamus* per inspection site, n_i = the number of wood objects or *Monochamus* adults sampled per inspection site and DP_i = the local level design prevalence. TSe_i = the test sensitivity for wood or *Monochamus* samples, i.e. the probability that the pest is detected in the laboratory analysis, given that it was present in the object from which the sample was taken. However, since the local level design prevalence was defined as the apparent prevalence, TSe was set equal to one.

The sensitivity of the annual surveys

The sensitivity of the annual surveys is the probability that the pest will be detected in an area (that may be an administrative region or the entire country) in a given year if it is present in the area at a prevalence equal to the design prevalence of the considered area.

The sensitivity of the annual surveys in the 15 administrative regions (group sensitivity, GSe) was first calculated separately for wood sampling (GSe_{wood}) and *Monochamus* trapping ($GSe_{Monochamus}$), which were then combined to obtain an overall sensitivity for each region (GSe). Then, the sensitivity of the annual surveys at the national level (system sensitivity, SSe) was obtained by combining the overall sensitivities of the annual surveys in the different regions (GSe).

The wood and *Monochamus* components of the group sensitivity were calculated, based on the binomial probability distribution, which is suitable for assessing the sensitivity of sampling from an infinite population (e.g. EFSA 2012):

$$GSe_{i,j} = 1 - (1 - DPr_j \cdot ISe_{i,j})^{N_{i,j}} \quad (5)$$

where i denotes either wood or *Monochamus* and j denotes the administrative region, $N_{i,j}$ = the number inspection sites in the region, with either wood sampling or *Monochamus* trapping, $ISe_{i,j}$ = the inspection sensitivity in the region for wood sampling or *Monochamus* trapping and DPr_j = the region level design prevalence. For the import-export survey, effective probability of infection (EPI , see equation 1) was used as the regional-level design prevalence.

Binomial distribution was considered appropriate for this assessment because the total area of the target population (i.e. risk areas) per administrative region (Table 3) was high compared to the number of inspection sites (Tables 1, 2). The rule of thumb is that a population can be considered infinite when the sample size is less than 10% of the total population size (Evans et al. 2000). This condition was fulfilled for all the regions for both wood and *Monochamus* samples.

The overall group sensitivity for each administrative region was obtained from:

$$GSe_j = 1 - \left(1 - GSe_{wood,j}\right) \cdot \left(1 - GSe_{Monochamus,j}\right) \quad (6)$$

which is the complement of the probability that, if PWN is present in the region at or above the design prevalence, it is not detected in wood sampling or *Monochamus* trapping.

Finally, the sensitivity of the annual surveys at the country level (system sensitivity, SSe) was calculated. For the import-export survey, it was obtained as the complement of the probability that, if PWN is present in Finland, it is not detected in any of the regions as follows:

$$SSe = 1 - \prod_{j=1}^{15} (1 - GSe_j) \quad (7)$$

where j denotes the administrative region. For the early detection survey, it was calculated as the sum of the regional-level sensitivities weighted by the relative probability of PWN invasion in the respective region as:

$$SSe = \sum_{j=1}^{15} GSe_j \cdot RP_j \quad (8)$$

where RP_j = the relative probability of PWN invasion in region j (see equation 2).

The probability of freedom from PWN based on evidence from several years

The probability of pest freedom is the probability that the prevalence of the pest is below the design prevalence if the pest is not detected in the surveys. It was estimated for each administrative region and for the entire country in a stepwise manner by progressively updating the estimate with evidence gained in the surveys in 2000–2018 using Bayes' theorem as follows:

$$Pfree_{t,j} = \frac{PriorPfree_{t,j}}{PriorPfree_{t,j} + \left[(1 - PriorPfree_{t,j}) \cdot (1 - Se_{t,j}) \right]} \quad (9)$$

(Martin et al. 2007), where j denotes the area considered (that may be an administrative region or the entire country), t = time, $PriorPfree_{t,j}$ = the prior probability of pest freedom and Se = the sensitivity of the survey. For the administrative regions, $Se = GSe_j$ (i.e. group sensitivity) and, at the national level, $Se = SSe$ (i.e. system sensitivity).

The initial prior probability of freedom, (i.e. the prior probability of freedom for the first time-step) was assumed to be 0.5 for all the regions and for the entire country, indicating that no information was available about the presence/absence of PWN before the surveys were started. To study the impact of this assumption on the probability of freedom achieved by 2018, the assessment was done also assuming an initial prior probability of freedom equal to 0.25.

For all the other time steps, the prior probability of freedom was calculated as the complement of the probability that a) the prevalence of the pest was above the design prevalence although it was not detected in the previous survey or b) the pest was introduced to the area after the previous survey as (Martin et al. 2007):

$$PriorPfree_{t,j} = 1 - \left[(1 - Pfree_{t-1,j}) + Pinv_{t,j} - (1 - Pfree_{t-1,j}) \cdot Pinv_{t,j} \right] \quad (10)$$

where $Pinv_{t,j}$ = the probability that the pest was introduced to the considered area after the survey conducted at time $t-1$.

The probability of invasion to the region j was calculated as:

$$Pinv_j = Pinv_{FINLAND} \cdot RP_j \quad (11)$$

where $Pinv_{FINLAND}$ = the probability of invasion to Finland and RP_j = the relative probability of PWN invasion to region j (see equation 2). Since the probability of PWN invasion to Finland was not known, a wide range of probabilities was studied. When presented in the results, the probability of invasion per year was translated to mean time between invasions to make the results easier to comprehend.

The parameters needed in the assessment

The number of wood objects and *Monochamus* sampled per inspection site

According to the survey guidelines of the NPPO of Finland, one wood sample could be composed of wood extracted from one or several trees or dead wood objects suitable for sampling. Unfortunately, information on the number of objects from which the samples were collected was not recorded. Based on discussions with inspectors who had undertaken the surveys, we concluded that the samples were typically composed of wood from a minimum of one, maximum of five and most often two objects.

These estimates were used to define a Pert probability distribution, which describes the probability distribution of the number of wood objects sampled per inspection site (n_{wood}). The lambda parameter, which defines the peakedness of the Pert distribution, was set to one, implying low confidence in the most likely estimate.

The number of *Monochamus* sampled per inspection site ($n_{Monochamus}$) in a given year and region was estimated by dividing the number of *Monochamus* caught by the number of traps used (Table 2). In the cases where *Monochamus* were caught by hand, each *Monochamus* was assumed to have been caught from a different inspection site.

The density of wood objects suitable for sampling

The density of wood objects suitable for sampling (D_{wood}) was estimated based on 1) the density of wood objects that are suitable for *Monochamus* breeding and 2) the propor-

tion of these objects that is suitable for sampling, i.e. the proportion of objects that have signs of *Monochamus* activity.

Since data from Finland were not available, the density of dead wood objects that are suitable for *Monochamus* breeding was estimated, based on data from Norway. According to Økland et al. (2010) in Norway, the number of dead wood objects suitable for *Monochamus* breeding per km² is most likely to be 288, whereas the minimum number is 166 and the maximum, 398. These estimates were used to define a Pert distribution describing the probability distribution of the density of dead wood objects suitable for *Monochamus* breeding (*obj*). The lambda parameter, which defines the peakedness of the Pert distribution, was set to one, implying low confidence in the most likely estimate.

The proportion of the *Monochamus* suitable dead wood objects (*obj*) that is suitable for sampling (*psam*) was not known and, therefore, it was described with a uniform distribution between 0.05 and 0.95. Finally, an estimate of the density of wood objects suitable for sampling (D_{wood}) was obtained by multiplying the two distributions (*obj* × *psam*) using Monte Carlo simulation. The median of the resulting distribution was 136 objects per km² and the 2.5th and 97.5th percentiles were at 19 and 309 wood objects per km², respectively.

The density of *Monochamus* adults

Two *Monochamus* species (*M. galloprovincialis* and *M. sutor*) are known to be widely present in Finland (Heliövaara et al. 2004; Rassi et al. 2015), but information about their density was not available. Therefore, the density of *Monochamus* adults ($D_{Monochamus}$) was estimated using the following data from Norway. The number of dead wood objects occupied by *Monochamus* per km² (*obju*) is most likely to be 28.8 (min 13.3, max 47.8), the number of *Monochamus* eggs laid per *Monochamus*-suitable dead wood object (*fobj*) is most likely to be 31 (min 6, max 88) and the proportion of *Monochamus* surviving from egg to egg-laying adults (*surv*) is most likely to be 0.25 (min 0.1, max 0.4) (Økland et al. 2010).

These figures were used to define the Pert distributions describing the probability distributions of the above-listed parameters (*obju*, *fobj* and *surv*). The lambda parameter of the Pert distributions was set to one, implying a low confidence in the most likely estimate. An estimate of the probability distribution of the density of *Monochamus* adults was obtained by multiplying these Pert distributions (*obju* × *fobj* × *surv*) using Monte Carlo simulation. The median of the resulting distribution was 266 adults per km² and the 2.5th and 97.5th percentiles were at 47 and 862 adults per km², respectively.

The size of the inspection sites

To convert the density of wood objects suitable for sampling (D_{wood}) and the density of *Monochamus* adults ($D_{Monochamus}$) to the number of wood objects suitable for sampling per inspection site (p_{wood}) and the number of *Monochamus* adults per inspection site ($p_{Monochamus}$), respectively, we needed to define the size of the inspection sites.

If the size of the inspection sites were defined based on the instructions given in the survey guidelines of the NPPO, it would have been, on average, 3 ha for the wood sampling (a sample per 2 ha or at least 200 m between samples) and 25 ha for the *Monochamus* trapping component of the survey (at least 500 m between traps). To control whether these sizes were appropriate considering the selected design prevalences, we checked if they were such that the number of infected individuals per inspection site at the design prevalence would be at least one. This was done by studying the estimated probability distribution of the density of wood objects suitable for sampling and that of the density of *Monochamus* adults.

The probability that the number of wood objects per inspection site was high enough was only 3.3%, whereas, for the number of *Monochamus* adults per inspection site, it was 97.6%. Hence, in the wood sampling component of the survey, the original size of the inspection site was too small, but in the *Monochamus* trapping component, it was adequate. We corrected this by adjusting the size of the inspection sites so that, at the apparent local level design prevalence, the number of infected individuals was at least one with a 95% probability. This adjusted size was 35 ha for the import-export survey and 63 ha for the early detection survey.

Adjusting the size of the inspection sites retrospectively was somewhat problematic. This is because some of the samples may have been collected so close to each other that, when the size of the inspection sites was increased, all samples did not actually represent the different inspection sites. However, this was deemed unlikely to have an impact on the results because the number of samples (Table 2) was very low compared to the total area covered by the surveys (Table 3).

Results

The PWN was not found in any of the 8,097 wood or 47 *Monochamus* samples collected and analysed in Finland in 2000–2018.

The sensitivity of inspections

The sensitivity of inspections was clearly higher for the wood sampling than for the *Monochamus* trapping component of the surveys (Table 5). In the import-export surveys, the median inspection sensitivity of the wood sampling component was 0.32 and, in the early detection surveys, it was 0.17. For the *Monochamus* trapping, the median inspection sensitivity was 0.00 in both types of surveys.

Table 5. The sensitivity of inspections of the import-export and early detection surveys. Only the regions and years with sampling activity were included. Abbreviations: ISe_{wood} = the inspection sensitivity of the wood sampling component, $ISe_{Monochamus}$ = inspection sensitivity of the *Monochamus* trapping component.

	Import-export			Early detection		
	Median	2.5%	97.5%	Median	2.5%	97.5%
ISe_{wood}	0.32	0.12	0.48	0.17	0.06	0.27
$ISe_{Monochamus}$	0.00	0.00	0.60	0.00	0.00	0.36

The sensitivity of the annual surveys

At the level of the administrative regions, the sensitivity of the annual surveys was rather low in most years and regions (Fig. 3). For the import-export surveys, it was at most 0.62 and, for the early detection surveys, it was at most 0.55 in all the regions and years with 97.5% probability.

At the national level, the sensitivity of the annual surveys was clearly higher than at the regional level for the import-export surveys, but not for the early detection surveys (Fig. 4). It was also clearly different for the two surveys types. For the import-export surveys, the sensitivity was at least 0.6 in 13 years with 97.5% probability, whereas, for the early detection surveys, the sensitivity was below 0.15 in 18 years, with 97.5% probability.

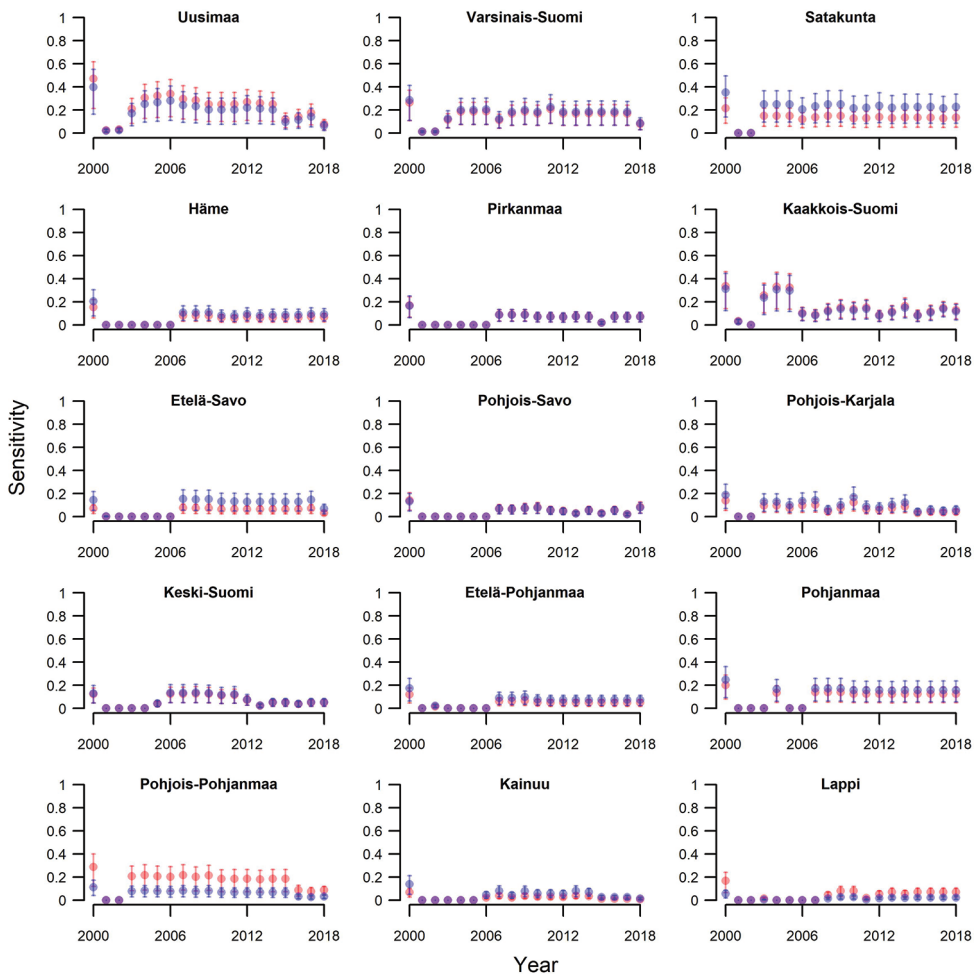


Figure 3. The sensitivity of the annual surveys in 2000–2018 in the administrative regions of Finland. The dots denote the medians and the bars the 95% confidence intervals of the assessment results. Red denotes the import-export surveys and blue denotes the early detection surveys.

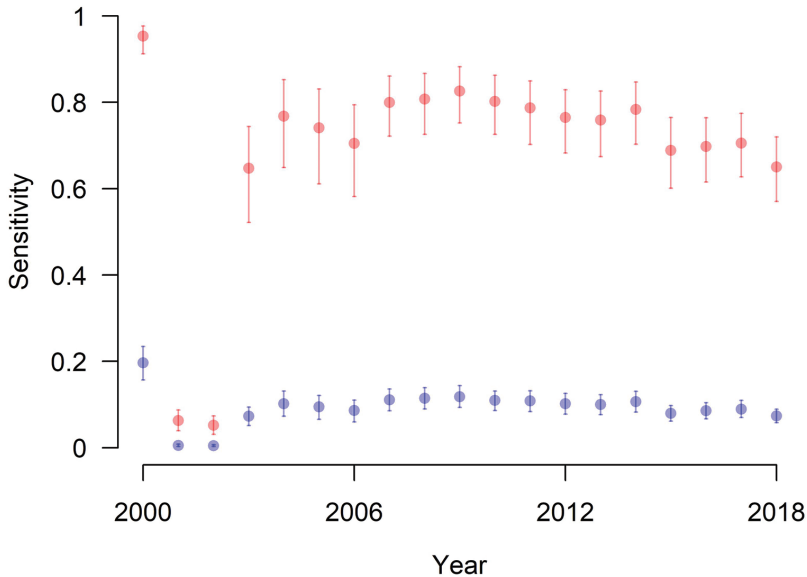


Figure 4. The sensitivity of the annual surveys in 2000–2018 in Finland. The dots denote the medians and the bars, the 95% confidence intervals of the assessment results. Red denotes the import-export surveys and blue denotes the early detection surveys.

The probability of freedom from PWN based on 19 years of surveys

The probability of pest freedom achieved by 2018 increased asymptotically with the mean time between PWN invasions (Figs 5, 6). In the administrative regions, the increase levelled out when the mean time between invasions was equal to 6–14 years and 6–17 years for the import-export and early detection surveys, respectively. At this levelling-out point, the probability of freedom was, at most, 0.05 lower than if the mean time between invasions was 100 years. At the national level, a similar levelling-out point occurred when the mean time between invasions was equal to 13 and 63 years for the import-export and early detection surveys, respectively.

The probability of pest freedom at the above-defined levelling-out point was rather high in many regions, both for the import-export and early detection surveys (Fig. 5). For both survey types, it was greater than 0.8 in five regions (with 97.5% probability). However, at the national level, the probability of pest freedom at the levelling-out point was clearly different for the two survey types (Fig. 6). It was 0.95 for the import-export and 0.73 for the early detection surveys (Fig. 6). The uncertainty of the assessment appeared to be low, since the probability distributions of the probability of pest freedom were narrow for both types of surveys (Figs 5, 6).

The used initial prior probability of freedom did not affect the probability of freedom achieved by 2018 in the import-export surveys, as it was similar for initial prior probabilities of freedom equal to 0.5 and 0.25 (Fig. 7). However, the probability of freedom achieved in the early detection surveys by 2018 was affected by the

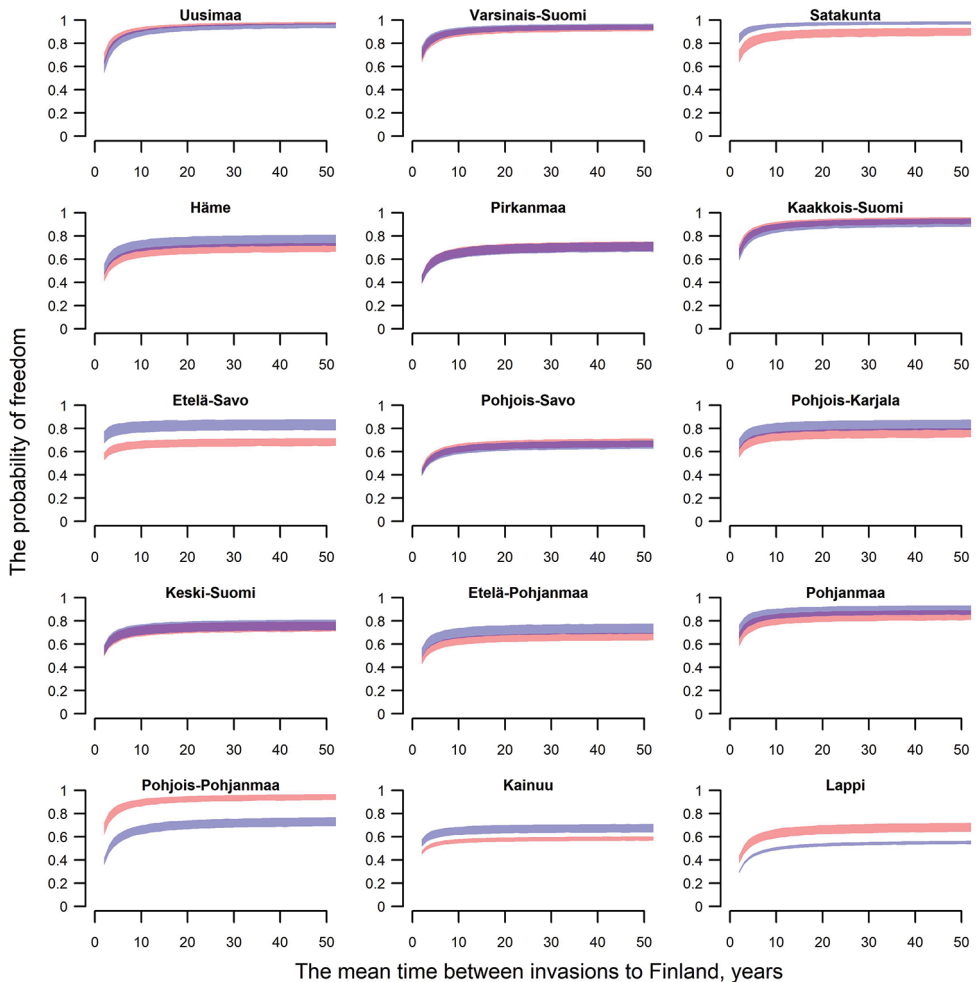


Figure 5. The probability of freedom from PWN achieved by 2018 in the administrative regions of Finland. The coloured areas show the 95% confidence intervals of the assessment results. Red denotes import-export surveys and blue denotes the early detection surveys.

initial prior probability of freedom that was used. It was clearly higher if the initial prior probability of freedom was 0.5 than if it were 0.25. This was true for all probabilities of invasion, except for those that were very high (Fig. 7).

Discussion

Reliable information about the distribution of quarantine pests is needed to prevent the pests from spreading with international trade. Additionally, if pest invasions are to be eradicated, they must be detected at an early stage, because, if the pest is widespread,

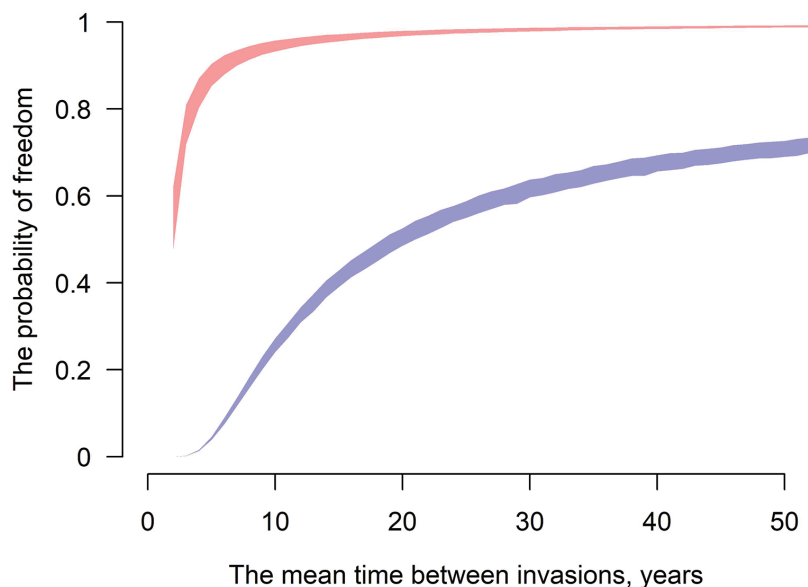


Figure 6. The probability of freedom from PWN achieved by 2018 in Finland. The coloured areas show the 95% confidence intervals of the assessment results. Red denotes the import-export surveys and blue denotes the early detection surveys.

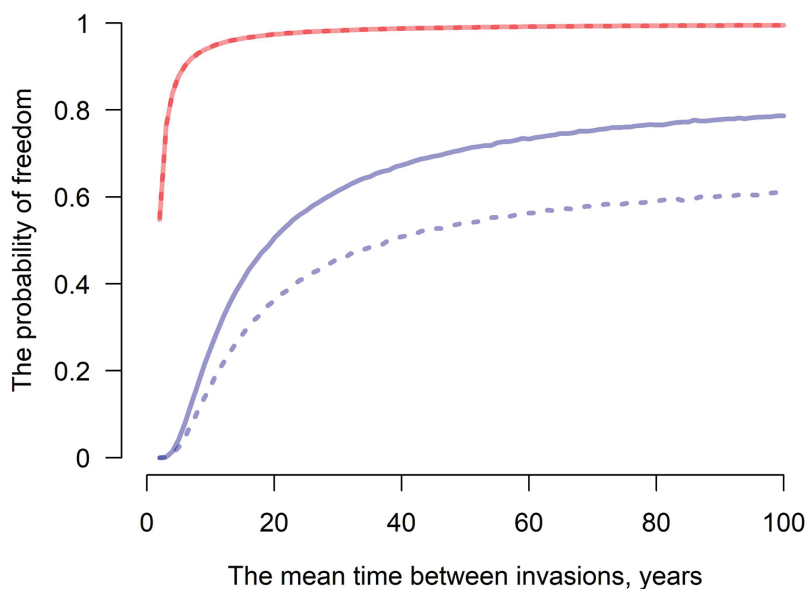


Figure 7. The probability of freedom from PWN by 2018 for two initial prior probabilities of freedom. The solid lines indicate the median of the assessment results when the initial prior probability of freedom was equal to 0.5 and the dashed lines indicate the results when the initial prior probability of freedom was equal to 0.25. Red denotes the import-export surveys and blue denotes the early detection surveys. Only one red line is visible, as the two lines overlap.

eradication is usually not feasible (Pluess et al. 2012a, b). To this end, all EU countries are required to conduct annual surveys for several quarantine pests, including PWN (European Council 2000, EU 2016). However, the sensitivity of these surveys has not yet been commonly analysed and, thus, it is not known if they are as useful for biosecurity as aspired.

Guidance on how to assess the sensitivity of annual surveys and the probability of freedom achieved in multiannual surveys is available (Cannon 2002; Martin et al. 2007; EFSA 2012, 2018), yet, so far, it has been widely applied mainly in the field of infectious animal diseases (e.g. Willeberg et al. 2011, but see, for example, Dominiak et al. 2011; Kean et al. 2015). Therefore, practical examples from the field of plant pests, such as the one presented in this paper, are essential for promoting a more objective analysis of official quarantine pest surveys and their impact on biosecurity.

The probability that Finland is free from PWN

The surveys support the assumption that PWN is not established in Finland. This is because the PWN was not found in any samples, although the sensitivity of the import-export surveys was rather high in many years and the probability of pest freedom achieved by 2018 was very high (≥ 0.95), unless the mean time between invasions was short (< 13 years). However, the surveys did not appear to be extensive enough to ensure early detection of PWN invasions. The sensitivity of the early detection surveys was very low in all years and the probability of freedom achieved by 2018 was rather low (< 0.73) unless the mean time between invasions was long (≥ 63 years).

The assessment seemed to be rather robust with respect to the parameters for which exact information was lacking (i.e. the density of wood objects suitable for sampling, the density of *Monochamus* adults and the number of wood objects from which a sample was collected). This is evident since the probability distributions of the sensitivity of annual surveys and especially those of the probability of pest freedom achieved by 2018 were rather narrow. Better data on the uncertain parameters would obviously improve the quality of the assessment, but acquiring such data does probably not deserve a high priority due to its minor impact on the outcome. It is noteworthy that aleatoric uncertainty (i.e. variation), which in these cases is inevitably large, cannot be reduced by more or better data.

Strictly speaking, the assessment of the probability of freedom did not cover the whole country, since the target population of the surveys was only the area with PWN host plants at 5 km radius from harbours, industrial areas and landfills (i.e. risk areas). However, these areas cover about 78% of the total area with PWN host plants in Finland (Table 3) and the probability of PWN infestation in the remaining remote locations is probably very low.

We did not find any published assessments of the sensitivity of PWN surveys done in other counties. However, Økland et al. (2010) assessed the probability with which the PWN surveys in Norway, together with the eradication measures proposed in the

Norwegian contingency plan for PWN, would result in successful eradication of a PWN outbreak. They did not report the sensitivity of the surveys for detecting a pre-defined pest prevalence (i.e. design prevalence), but they did report that the probability with which a PWN outbreak would be detected during the first years of invasion was extremely low (0.00013 and 0.011 for the 1st and 4th year, respectively).

Quantitative estimates of the probability of invasion are needed

Being able to accumulate evidence for pest freedom from consecutive surveys would be very useful. For both survey types, the support for the assumption that PWN is absent from Finland was much stronger if the evidence from all the years were pooled than when the surveys done in different years were analysed separately. This was true for all except high probabilities of invasion.

To pool evidence from consecutive surveys, a quantitative estimate of the probability of pest invasion is needed. However, very rough estimates apparently may be sufficient because, when the mean time between PWN invasions was above a certain level, its increase had only a very small impact on the probability of pest freedom.

A quantitative estimate of the probability of PWN invasion to Finland is not available, although the probability of PWN entry to new areas in the European and Mediterranean countries has been assessed as “considerable” and the probability of PWN establishment as “highly likely” (EPPO 2009). Moreover, Douma et al. (2017) assessed the exposure of European pines to PWN via the trade of wood and they estimated that in Finland, at most, approximately 1.2 PWN per year come into contact with a host tree. However, to be able to translate this figure into probability of invasion, the probability that such a contact results in the establishment of a PWN population should be assessed too.

Most pest risk assessments are qualitative and, therefore, quantitative estimates of the probability of invasion are available only for some pest species/area at risk combinations, such as *Sirex noctilio* and North America (Koch et al. 2009; Yemshanov et al. 2009; 2010). EFSA Panel on Plant health (EFSA PLH Panel) has recently published a protocol for quantitative pest risk assessment (EFSA PLH Panel 2018a), which has been, this far, applied to nine assessments (EFSA PLH Panel 2016a; 2016b; 2016c; 2016d; 2017a; 2017b; 2017c; 2017d; 2018b), some of which report estimates that could be translated to probability of invasion per year. Although the assessments were done at the EU level, they could probably be used to obtain an indication about the order of magnitude of the probability at the national level too.

Defining meaningful design prevalence is crucial

Defining design prevalences with care, so that they reflect the aims of the survey, is central. Unfortunately, very little guidance is available for defining design prevalences for

quarantine pests. Martin et al. (2007) advise that the design prevalences for infectious animal diseases should be based on international standards, requirements of the trading partners, political considerations, availability of resources and/or biological plausibility. The list is relevant also for quarantine pest surveys if the aim of the survey is to justify import requirements and to facilitate export. However, if the aim of the survey is to detect pest invasions early enough to enable successful eradication of outbreaks, only the last two (availability of resources and biological plausibility) are relevant.

The international standard for phytosanitary measure that sets the requirements for surveillance (FAO 2018) encourages NPPOs to report the minimum pest prevalence that a survey is aiming to detect (i.e. design prevalence) and the probability with which it is expected to succeed in this aim (i.e. sensitivity). However, the standard comments neither on the appropriate levels of those parameters nor on how they should be defined. Additionally, EU legislation leaves the definition of the design prevalence to the member states, although it requires that sound scientific principles are used and timely detection of the pests is ensured with a high degree of confidence. EFSA is currently preparing survey guidelines for several quarantine pests (EFSA 2018), which will hopefully aid NPPOs in defining design prevalences.

We defined the local-level design prevalences of PWN, based on the prevalence of a closely-related species, *Bursaphelenchus mucronatus*, in the samples collected in the PWN survey. Thus, all the biases in the sampling process of *B. mucronatus* and PWN were the same, which was perfect for our purpose. However, the reported prevalence of *B. mucronatus* cannot be expected to correspond to the prevalence of *B. mucronatus* in standing trees, because the sampling was targeted at material that had signs of *Monochamus* activity.

We defined the regional- and national-level design prevalences for the early detection surveys based on article 7 of the EU emergency measures for PWN (EU 2012), which allows member states to refrain from attempting eradication if the diameter of the infested area is more than 20 km. However, it is not clear if such a large infestation could be eradicated with the resources available for delimiting the infested area and conducting the eradication measures.

Misinformed initial prior probability of freedom may distort the assessment

The prior probability of freedom at the first time-step (i.e. the initial prior probability) should be in line with the probability of invasion, unless reason exists to assume that the probability of invasion was different before the survey was initiated. In other words, if the probability of invasion is assumed to be high, assuming the initial prior probability of freedom is low is not logical and vice versa.

This appeared to be worth considering even when using a seemingly uninformative initial prior probability of freedom equal to 0.5. In the early detection survey, in which the sensitivity of annual surveys was low, the initial prior probability of freedom had an impact on the probability of freedom even after 19 years of surveys, unless the probability of invasion was very high.

This shows that, if the sensitivity of the survey is low, the initial prior probability of freedom can have an impact on the probability of freedom for several years. Thus, in such cases, the results from the first years of surveys should be interpreted with caution if the initial prior probability is uncertain. This is especially relevant if the trend in the probability of freedom is decreasing because, in such cases, the results for the first years are likely to be too optimistic.

Statistical analysis should be considered already when planning surveys

Some complications encountered in the current assessment emphasise the importance of proper survey planning and indicate some of the issues that one should be aware of when planning surveys. First, risk areas should be defined so that they cover a sensible proportion of the total area at risk. Otherwise, the value of classifying areas according to risk is compromised. In the Finnish PWN survey guidelines, the definition of risk areas was such that they covered most of the area with PWN host plants and, therefore, the risk-based survey design could not be used in the assessment. The probability of freedom from PWN achieved with the surveys would probably be higher if a risk-based design were used.

Second, local design prevalence should be defined and the density of objects suitable for sampling should be estimated before the area covered by one inspection (inspection site) is defined. This is because the size of the inspection site should be such that, at the local level design prevalence, the number of infected objects per inspection site is at least one. In the Finnish PWN survey guidelines, the area covered by one inspection was so small that, at the local level design prevalence, the number of infested wood objects was less than one and, therefore, we had to redefine the size of the inspections site for this assessment.

Conclusions

The PWN surveys conducted in Finland in 2000–2018 appeared to support the assumption that PWN is not present in Finland, but they did not seem extensive enough to ensure early detection of PWN invasions. Without corresponding assessments, it is not possible to tell if, for example, the PWN surveys in the other EU countries have been any better or how much the surveys of other quarantine pests benefit biosecurity.

The efficiency of the surveys could probably be improved by revising the definition of risk areas (e.g. to 2 km radius from harbours and industrial areas) and by optimising the number of inspected sites versus the number of samples collected per inspected site. However, without a thorough assessment, it is impossible to know if such revisions could improve the efficiency enough, i.e. so much that PWN outbreaks would be detected, with a high degree of confidence, early enough to facilitate eradication.

To enable analysis of pest freedom, based on multiannual surveys, quantitative estimates of the probability of invasion are needed, but rather rough estimates may be sufficient. Furthermore, methods for determining meaningful design prevalence, especially for early detection surveys are needed. Ideally, the design prevalence in early detection surveys should represent the area from which eradication of the pest is feasible.

To learn whether the current quarantine pest surveys, in the EU and elsewhere, are as beneficial for biosecurity as aspired, we need many more examples of the sensitivity that is, in practice, achieved in the surveys. Otherwise, the only result of the surveys may be a false sense of biosecurity.

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Supplementary material 1

readme.txt

Authors: Salla Hannunen, Juha Tuomola

Data type: instructions

Explanation note: Instructions on how to run the R-scripts needed to make the assessments presented in “Assessing the probability of freedom from pine wood nematode based on 19 years of surveys” by Hannunen and Tuomola.

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Link: <https://doi.org/10.3897/neobiota.58.38313.suppl1>

Supplementary material 2

Sensitivity.R

Authors: Salla Hannunen, Juha Tuomola

Data type: R code

Explanation note: This script calculates the sensitivity of the annual surveys in 2000-2018 for all the administrative regions and Finland and plots them as figures.

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Link: <https://doi.org/10.3897/neobiota.58.38313.suppl2>

Supplementary material 3

Probability_of_freedom.R

Authors: Salla Hannunen, Juha Tuomola

Data type: R code

Explanation note: This script calculates the probability of freedom achieved by 2018 for a range of probabilities of invasion for all the regions and Finland and plots them as figures.

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Link: <https://doi.org/10.3897/neobiota.58.38313.suppl3>

Supplementary material 4

Data.R

Authors: Salla Hannunen, Juha Tuomola

Data type: R code

Explanation note: This script contains the data used in the assessment.

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Link: <https://doi.org/10.3897/neobiota.58.38313.suppl4>

Supplementary material 5

Sensitivity_function.R

Authors: Salla Hannunen, Juha Tuomola

Data type: R code

Explanation note: This script contains a function that returns the sensitivity of the annual surveys in 2000–2018 for all the regions and Finland in one array.

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Link: <https://doi.org/10.3897/neobiota.58.38313.suppl5>

Supplementary material 6

Probability_of_freedom_function.R

Authors: Salla Hannunen, Juha Tuomola

Data type: R code

Explanation note: This script contains a function that returns the probability of freedom achieved by 2018 for a range of probabilities of invasion for all the regions and Finland in one array.

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Pathologists and entomologists must join forces against forest pest and pathogen invasions

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Abstract

The world's forests have never been more threatened by invasions of exotic pests and pathogens, whose causes and impacts are reinforced by global change. However, forest entomologists and pathologists have, for too long, worked independently, used different concepts and proposed specific management methods without recognising parallels and synergies between their respective fields. Instead, we advocate increased collaboration between these two scientific communities to improve the long-term health of forests.

Our arguments are that the pathways of entry of exotic pests and pathogens are often the same and that insects and fungi often coexist in the same affected trees. Innovative methods for preventing invasions, early detection and identification of non-native species, modelling of their impact and spread and prevention of damage by increasing the resistance of ecosystems can be shared for the management of both pests and diseases.

We, therefore, make recommendations to foster this convergence, proposing in particular the development of interdisciplinary research programmes, the development of generic tools or methods for pest and pathogen management and capacity building for the education and training of students, managers, decision-makers and citizens concerned with forest health.

Keywords

Capacity building, detection, disease, exotic, fungi, forest health, identification, insects, interdisciplinarity, management

The United Nations General Assembly declared the year 2020 as the International Year of Plant Health (IYPH). We take this unique opportunity to affirm that the phytosanitary protection of forests, which is essential for the maintenance of their functions (e.g. climate regulation, wood production, biodiversity reservoir) and, ultimately, for human well-being, requires the joint effort of entomologists and pathologists to prevent or manage severe pest and pathogen problems. In a year characterised by a global threat to human health from the COVID 19 coronavirus pandemic, attention to plant health could be considered derisory. We believe, however, that plants face similar threats and that trees and forests, in particular, play an essential role in providing humans with important services that fit within the concept of “One health” (Xie et al. 2017), because humans will suffer if trees disappear from the landscape.

Throughout the article, we will use as a definition of “pests” insect herbivores that inflict damage to trees and as “pathogens” microorganisms that cause disease to trees, including fungi, oomycetes, bacteria, viruses and nematodes.

Forests under biotic threat

Due to global change, the world's forests are exposed to unprecedented threats from biotic hazards (Simler-Williamson et al. 2019). The increase in volume and acceleration of global trade and travel has boosted the risk of invasion by non-native species into forests (Roy et al. 2014). On all continents, the number of non-native forest insects (Hurley et al. 2016; Brockerhoff and Liebhold 2017) and pathogens (Santini et al.

2013; Ghelardini et al. 2017) that have become established outside their natural range has increased dramatically and this trend shows no signs of levelling off (Seebens et al. 2017). Currently, the greatest damage in forests is often caused by these invasive alien species, including insect pests, such as the Eurasian woodwasp and its associated decay fungus (Hurley et al. 2007), the emerald ash borer (Poland et al. 2006), the polyphagous shot hole borer and its associated fungal pathogens (Paap et al. 2018), the Asian longhorn beetle (Haack et al. 2010) and pathogens, such as the causal agents of sudden oak death (Davidson et al. 2003), ash dieback (Gross et al. 2014), rapid ohia decline (Barnes et al. 2018), Dutch elm disease or the pine wilt disease (Soliman et al. 2012), the latter two being vectored by insects.

Many aspects of climate change promote the emergence of native forest pests and pathogens, foster epidemics and trigger outbreaks in a number of ways. Warmer temperatures may favour winter survival and accelerate the rate of development of many fungi and insects (Robinet and Roques 2010; Santini and Ghelardini 2015; Pureswaran et al. 2018; Jactel et al. 2019; Lehmann et al. 2020). A higher number of generations per year, or increased reproduction rates in univoltine species, results in accelerated population growth. Increase in winter temperatures releases constraints on year-to-year survival of some insect and pathogen species (Marçais et al. 1996; Aguayo et al. 2014), leading to range expansions towards higher elevation and latitudes in the northern hemisphere (Bergot et al. 2004; Battisti et al. 2005; Lehmann et al. 2020). In addition to the warming trend, increasing numbers of extreme events are occurring (IPCC 2012), which also contribute to these epidemics. More frequent or severe droughts lead to water stress on trees (Greenwood et al. 2017), making them more susceptible to opportunistic insect pests and pathogens (Desprez-Loustau et al. 2006; Jactel et al. 2012). Intense windstorms (Gardiner et al. 2013) provide sudden substantial increases in breeding substrates for bark beetles and substrates for fungal infection, which can build up large populations and eventually kill many standing trees (Seidl et al. 2017). Large and severe fires associated with warm and dry conditions, more frequent in a warming climate, may also favour insect outbreaks (Halofsky et al. 2020) and, conversely, trees killed by pests and pathogens may fuel forest fires (Jenkins et al. 2008). Climate change can affect upper trophic levels in different ways, leading to idiosyncratic responses. Parasitoids, for example, may respond positively to temperature increases (Péré et al. 2013), which may explain the decrease in damage observed in some key pest species (Lehmann et al. 2020). Furthermore, climate change, not only provides improved opportunities for many native species, but also invasive alien species from warmer regions (Walther et al. 2009).

Both alien and emerging native forest pests and pathogens have had and will continue to have profound impacts on forest vitality and the economy (Aukema et al. 2011; Ramsfield et al. 2016; Stenlid and Oliva 2016). Yet, the need for forest ecosystems to meet the increasing global demand for biosourced materials and products, to preserve biodiversity (Myers et al. 2000), to contribute to climate change mitigation (Griscom et al. 2017) and the provision of other forest ecosystem services has never been greater. This increasing demand can itself be a factor contributing to new disease

risks, for example, with the development of extensive plantations of exotic trees, where introduced tree species are exposed to resident pests and pathogens to which they have not evolved resistance (Wingfield et al. 2008; Burgess and Wingfield 2015). Moreover, intensification of forestry practices is often associated with reduced stand heterogeneity, especially reduced tree species and genetic diversity (with clonal forestry at the extreme), which may strongly increase pest and pathogen risk (Desprez-Loustau et al. 2016; Persoons et al. 2017; Jactel et al. 2017).

There is clearly an urgent need to develop a common framework to understand insect and pathogen invasions and to develop methods for forest protection that are effective against both tree pests and pathogens. However, forest entomologists and forest pathologists have traditionally followed different conceptual and methodological approaches to understand the epidemiology of pests and pathogens (Wingfield et al. 2017) and they have developed different management approaches for their subjects of study (Raffa et al. 2020). Consequently, the number of scientific papers simultaneously addressing insect pests and fungal pathogens is low. This can be illustrated using the published content from two major journals taken as examples and which specialise separately in pathology and entomology, respectively: *Forest Pathology* (previously *European Journal of Forest Pathology*) and *Agricultural and Forest Entomology*. An average of 11% of papers from the last thirteen years of *Forest Pathology* mentioned insects in their title, key words or abstract (Fig. 1A) and 10% of papers, published since the first issue of *Agricultural and Forest Entomology*, mentioned pathogens (Fig. 1B). In both journals, the number of papers has increased with time while the proportion of papers intersecting the disciplines of pathology and entomology remains low and stable.

Data were obtained from a keyword search of the Web of Science database on 8 March 2020, using the following searches “[((forest pathology) or (European journal of forest pathology)) AND TOPIC: ((insect* or pest or herbivor* or beetle or scolyt* or moth)))] and [(agricultural and forest entomology) AND TOPIC: (forest or tree or oak or pine or birch or spruce or fir or beech or maple) AND TOPIC: (fung* or (fungal pathogen*) or (fungal disease) or phytophthora)]”.

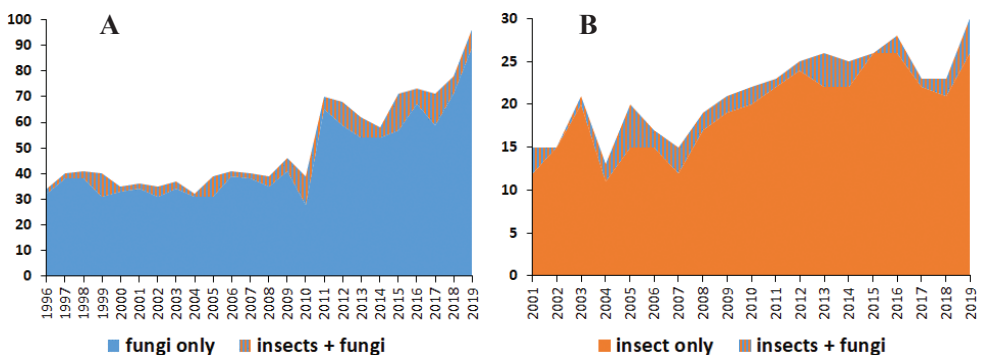


Figure 1. Temporal trend of the number **A** of articles dealing only with forest fungi or with both forest fungi and insects in the (*European Journal of Forest Pathology*) (1996–2019) and **B** of articles dealing only with forest insects or with both forest fungi and insects in the journal *Agricultural and Forest Entomology* (2001–2019).

Hereafter, we explain how the conservation of forest health would greatly benefit from more effective cooperation between forest pathologists and forest entomologists and suggest ways to achieve this outcome.

Preparedness and border surveillance

An essential step in the prevention and control of forest pest and pathogen problems is their recognition as potentially damaging agents and preventing their arrival. Ideally, insects and microbes that have the potential to become pests and pathogens should be identified and the damage they cause characterised, before they are introduced to new areas, because this would provide time to develop and implement measures for detection and management. As invasive organisms are frequently associated with plant trade, a commodity risk assessment may be useful and it was recently adopted as a strategic approach by the European Union, with pathologists and entomologists in the same working group (EFSA 2019). Sentinel plantings in exporting countries provide excellent resources for early identification of plant pests and pathogens at high risk of causing damage should they become introduced. Consisting of woody species that are native to importing countries, sentinel plantings can serve to identify the pests and pathogens of highest potential to impact trees in the importing country (Eschen et al. 2019). Studies of tree health in these facilities also represent an ideal opportunity for collaboration between entomologists and pathologists.

Improved knowledge of the pathways of movement and entry of alien organisms is a key step towards improved strategies for preventing arrival of these organisms through quarantine measures. Recent studies have shown that many pathways by which alien forest pests and pathogens move worldwide are shared amongst these organisms, being mainly associated with trade in live trees or germplasm and transport of wood packing material (Liebhold et al. 2012; Ghelardini et al. 2017; Meurisse et al. 2019). Identification of these pathways is crucial for the adoption of measures, such as phytosanitary treatments, to prevent introductions (Allen et al. 2017). Research identifying the wood packaging and live plant invasion pathways has led to global implementation of phytosanitary standards such as ISPM 14 (International Standards For Phytosanitary Measures No. 14, 2019) “The use of integrated measures in a systems approach for pest risk management” and ISPM 15 (2019) “Regulation of wood packaging material in international trade” resulting in tangible decreases in risks of new invasions (Kenny 2002; Leung et al. 2014). However, further work is needed to identify emerging pathways common to pests and pathogens, as well as strategies for mitigating the impacts of these pathways.

New technologies for alien forest pests and pathogens detection and identification

Detection of pests and pathogens at ports of entry is complicated by the volumes of material that are imported and generally a lack of capacity of quarantine officers. Many

emerging technologies could substantially improve this situation (Luchi et al. 2020). For example, many forest insects and pathogenic fungi emit volatile organic compounds sufficiently characteristic to indicate their presence (Nixon et al. 2018). Detection devices for such volatile compounds could be developed (e.g. e-nose), installed in containers at their point of departure and automatically checked at their point of arrival, to help with the screening of large volumes of commodities (Poland and Rassati 2019).

Most alien insect pests and pathogens that cause damage in invaded areas were not known as causes of damage, or even described, in their area of origin (Roques et al. 2015; Burgess and Wingfield 2015). Moreover, many insects and fungi can hardly be identified at species level on the basis of morphology alone, making it difficult to distinguish a potential introduced organism from a closely-related native species, as exemplified by *Hymenoscyphus fraxineus*, the causal agent of ash dieback (Gross et al. 2014) or the brown spruce longhorn beetle (*Tetropium fuscum*) which was not recognised as an exotic in Canada, because of morphological similarity to the native *Tetropium cinnamopterum* (Ramsfield 2016). It is, therefore, essential to develop molecular tools that will allow detection and identification of potentially invasive alien species to be able to set up measures to eradicate them at an early stage (McTaggart et al. 2016). Historically, molecular methods of identification have been more developed for fungal pathogens because it is especially difficult to recognise species, based on morphological features of the fungal spores (Taylor et al. 2000; Pashley et al. 2012; Steenkamp et al. 2018). However, the same difficulties apply to the recognition of insect immature forms such as larvae. Cooperation between forest entomologists, pathologists and molecular biologists would accelerate the development of pipelines for the rapid identification of these unknown organisms (Feau et al. 2011; Malacrinò et al. 2017). In addition, emerging molecular methods, based on metabarcoding, may allow the characterisation of entire communities, which offers great prospects for surveillance of both pests and pathogens, based on environmental samples (e.g. eDNA; Aguayo et al. 2018; Piper 2019).

Another approach that should be shared by plant pathologists and entomologists is risk modelling. Quantitative pest and pathogen risk assessment is recommended, because it allows various risk reduction options to be tested in order to enable decision support schemes (EFSA PLH Panel 2018) while quantifying uncertainty levels. This approach follows the same steps as those of the invasion process (i.e. arrival, establishment, spread and impact) and, therefore, makes it possible to prioritise the areas or products to be monitored as a matter of priority, which ultimately optimises early detection (Robinet et al. 2012; Douma et al. 2015; Gottwald et al. 2019). Clearly, forest pathologists and entomologists can work together using such a methodology for forecasting and their cooperation will help to take into account multiple hazards to strengthen the conclusions of these quantitative risk analyses.

Post-border surveillance

Despite efforts to prevent potentially damaging species from arriving, many such organisms will evade detection and potentially establish alien populations. Early de-

tection of nascent populations is critical to the success of attempts to eradicate such populations and integrated surveillance programmes therefore play a key role in national biosecurity programmes (Coulston et al. 2008; Pluess et al. 2012; Liebhold et al. 2016). Surveillance for arrivals of alien forest pests and pathogens should focus in high-risk areas, such as urban and peri-urban forests close to industrial and commercial areas and near ports and airports (Branco et al. 2019). Characterisation of geographical variation in invasion risk and optimal allocation of surveillance resources across that variation is critical to the success of surveillance programmes (Epanchin-Niell 2017).

The isolation and identification of pheromones and other semiochemicals has played a key role in providing trapping technologies used in insect surveillance programmes (Poland and Rassati 2019). Combining multiple lures, targeting various pest species in a single trap, holds great potential in the development of integrated pest surveillance programmes (Brockhoff et al. 2013). Spore-trapping, stream baiting and other technologies also hold potential for integration of tree pathogen detection in national biosecurity programmes (Sutton et al. 2009; Botella et al. 2019). Increasingly, citizen science projects have become important for detection and surveillance in many countries. Importantly, the efficacy of these projects, as well as the confirmation of records received, requires expert backing from the disciplines of both entomology and plant pathology. This is particularly true in the case of web applications that require the public to report any form of damage observed in trees, as, for example, in the Silvalert (www.silvalert.net) and Treealert (<https://treealert.forestresearch.gov.uk>) projects. Strong communication and data sharing within and between countries is essential to prepare for emerging threats to forests. The European Union EUROPHYT platform is a leading example of such best practice for official notifications and rapid alerts, as are the databases provided by CABI and EPPO.

Interactions between organisms on host trees

For many pathogens, transmission and/or introduction into the host by an insect vector is essential for infection and spread (Wingfield et al. 2016; Santini and Battisti 2019). Insect vectoring is the main if not sole way of dissemination of many important vascular pathogens, such as *Xylella fastidiosa*, the cause of Bacterial Leaf Scorch, vectored by leafhoppers and froghoppers (Landa et al. 2020), *Ophiostoma novo-ulmi*, the agent of Dutch Elm Disease, vectored by elm bark beetles (McLeod et al. 2005) and the pine wilt nematode, *Bursaphelenchus xylophilus*, vectored by *Monochamus* longhorn beetles (Sousa et al. 2001). In the case of bacteria, insects may serve as alternative primary hosts (Nadarasah and Stavrinos 2011).

It is increasingly acknowledged that insects and microbes interact in and on their host trees. Insect infestation can predispose trees to attack by fungal pathogens, increasing damage caused by the pathogens and enabling weaker pathogens to attack hosts (Xi et al. 2018). Some forest insects are known to carry various species of fungi that they use as symbionts for larval development (Ramsfield 2016), “cultivate” and use for food in the galleries they form in the tree (e.g. ambrosia beetles) or to overcome

the induced defences of colonised trees (e.g. bark beetles) (Six and Wingfield 2011). In some cases, fungal associates of beetles are tree pathogens (Hulcr et al. 2011), which can explain the high rate of tree mortality recently caused by the massive attacks of the polyphagous shot hole borer (Paap et al. 2018). Diseases associated with ambrosia and bark beetles and their symbiotic fungi are amongst the most important emerging problems affecting tree health in the last century (Ploetz et al. 2013, Fig. 2).

In contrast with their mutualistic relationships, insects may be natural enemies of pathogenic fungi, with some species being putative obligate mycophages (Dillen et al. 2017). Additionally, fungal tree infection by biotrophic pathogens and endophytes can reduce insect performance on challenged trees (Fernandez-Conradi et al. 2018). Fungi may have direct toxic effects on insects, being entomopathogens (Dowd 2000) or indirect tree-mediated effects through reduced nutritional quality or induction of systemic defences against herbivores. It is known that plants use cost-effective inducible defences to protect against insects and pathogens, whilst the latter have developed mechanisms to overcome and/or manipulate those defences to their benefit. Both insects and fungi can trigger host plant defence responses through the biochemical pathways of jasmonic acid (JA), salicylic acid (SA) and ethylene (ET). Many examples exist where JA and SA can interact antagonistically (Thaler et al. 2012) and recent insights suggest they could also interact synergistically (Liu et al. 2016). During multi-attack events, the activation of defences towards one attacker can increase or reduce susceptibility to the other (Vos et al. 2013; Castagnéyrol et al. 2018). Although our knowledge regarding plant defences in crop systems has improved in recent years, the study of defence mechanisms against both insects and pathogens in forest trees is only beginning to emerge.

Symbiosis between trees and mycorrhizae can modify tree physiology and tree-insect interactions (Koricheva et al. 2009), with effects depending on the feeding guild

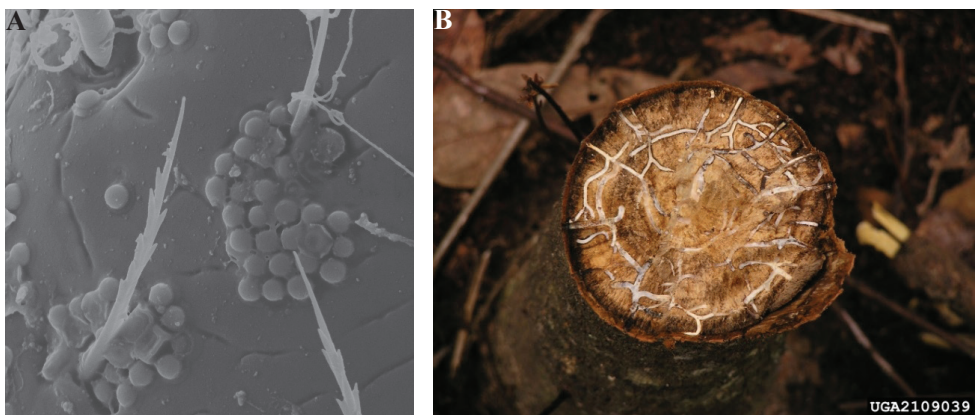


Figure 2. Examples of interactions between forest insects and fungi **A** detail of the abdomen of the ambrosia beetle *Xyleborinus saxesenii* (Ratzeburg, 1837) from below with fungal spores (Courtesy of Peter Biedermann, University of Freiburg, Germany) **B** mycelium filling the galleries of the ambrosia beetle *Xyleborus glabratus* (Eichhoff, 1877) (Courtesy of James Johnson, Georgia Forestry Commission, Bugwood.org).

of the insect and the type of mycorrhizae. Likewise, interactions between mycorrhizae and plant pathogens should not be overlooked, as rhizosphere fungi have the potential to exclude, outcompete or enhance the defence system of plants to more effectively respond to invading pathogens (Selosse 2014). However, it remains largely unknown how the complex interactions between the tree and its microbiome, which forms the holobiont, affect tree susceptibility to pests and pathogens (Vivas et al. 2015; Mishra et al. 2020). This necessitates a more holistic approach to understanding of biotic interactions involving insects, fungi, oomycetes, viruses and bacteria at both the individual tree and forest levels and their consequences for forest health (Naidoo et al. 2019).

Control measures of forest pests and pathogens

Once they have attacked a tree, both insects and pathogens are often difficult to locate for treatment. Most species are inconspicuous, living under the bark or within tissues, such as bark beetles and leaf miners or vascular fungi and root pathogens. External feeders (e.g. defoliators) or diseases (e.g. leaf rusts) are located in the crowns of trees that are tens of metres above the ground. This makes it difficult and often ineffective to apply insecticides and fungicides. Indeed, pesticides are typically not effective at controlling forest insect and disease outbreaks at a regional scale (Liebhold 2012). The negative effects of pesticides on human health and the environment and the risk of pests and pathogens developing resistance to them, are receiving more attention. This has led to their rejection by the public and bans on their use in forests by the authorities, as has occurred with neonicotinoids in Europe (Jactel et al. 2019). There is consequently a common need for alternative control methods against tree pests and pathogens.

Preventative control measures should be favoured and previous studies have shown that adapting forest management to reduce stand susceptibility is the most promising approach. For example, selecting tree species suitable for future pedoclimatic conditions, as well as initial fertilisation and regular thinning, are methods that can increase the vigour of individual trees and could improve their resistance to secondary insects and pathogens (Jactel et al. 2009). Increasing tree species diversity improves forest resistance (i.e. associational resistance) by various bottom-up and top-down mechanisms such as reducing the likelihood of propagules reaching host trees and promoting the control of pests and pathogens by their natural enemies (Jactel et al. 2017; Grosdidier et al. 2020). However, the direction and magnitude of the effect of host species diversity on disease incidence (the so-called “dilution effect” when negative) remains controversial and contrasting evidence exists (Liu et al. 2020). An improved understanding of the effect of biodiversity on forest vulnerability to damaging biotic agents and joint research between entomologists and pathologists are required to identify the silvicultural and land use management practices that could effectively reduce the impact of multiple damaging agents.

Where alien pests and pathogens become established and multiply too rapidly in an area to be eradicated, then the priority shifts to preventing or slowing their further

spread. Common features have been identified that influence the invasibility (resistance to invasion) of forest landscapes by non-native insects and pathogens. In particular, there is mounting evidence that a homogeneous forest landscape with a high proportion of the main host species, in the form of large monocultures or large connected patches, would favour the rapid spread of forest pests and pathogens (Condeso and Meentemeyer 2007; Morin et al. 2009; Rigot et al. 2014; Haas et al. 2016; Hudgins et al. 2017; Prospero and Cleary 2017). To further develop and challenge our understanding of these effects and better predict areas at higher risk of contagion, it is important to develop spread models that address both insects and pathogens and to test the simulations in realistic forest landscapes (Robinet et al. 2019; Barron et al. 2020). Although the processes of natural dispersal of organisms differ between insects and fungi, mainly active dispersal by flight for the former and passive dispersal via wind, rain or vectors for the latter, human-assisted dispersal and the barriers to dispersal are similar for both. These are mainly landscape composition (proportion of host and non-host habitats) and fragmentation over short distances and population density and trade networks for human-assisted spread over long distances (Hudgins et al. 2017). As is true for surveillance and early detection, generic modelling frameworks could be developed for both insect pests and pathogens in order to better understand the potential spread of biological invasions, optimise monitoring systems and manage the landscape to reduce their spread rates and their impacts. Finally, as a control measure, classical biological control has been much studied and applied to manage pests and less so to control pathogens (but see Rigling and Prospero 2018). This approach certainly deserves more research in forest pathology, especially against invasive pathogens.

Conclusions

We have argued that to improve forest protection, insects and pathogens should be considered collectively. In addition, although traditionally considered separate disciplines, many tools and conceptual frameworks can and should be shared between forest entomology and pathology. To further facilitate such collaboration and increase its benefits, we make the following recommendations:

1. Research policy

- An interdisciplinary approach including entomology and plant pathology, but also economics and social sciences, should be encouraged in all research projects dealing with the adaptation of forests to global change and, in particular, with the risks to forest health.
- Specific research topics involving interactions between forest insects and pathogens should be prioritised, such as insect-vectored diseases (e.g. ambrosia beetles) and physiological host tree responses to multiple biotic stresses (e.g. priming effects, cross-talks between defence pathways).

2. Research implementation and development

- Innovative tools should be designed together by plant pathologists and entomologists, such as pipelines for high-throughput molecular species identification, artificial intelligence in smart sensors for detection of non-native organisms (e.g. detecting VOCs) and generic models for risk analysis and spread prediction.
- Science-based guidelines should be developed to provide new sustainable forest management alternatives aimed at reducing the vulnerability of stands to both pests and diseases.
- Forest entomologists and forest pathologists should collaborate to improve biosecurity strategies, such as those targeting the movement of damaging organisms associated with live plants and wood products.

3. Capacity building

- Forest entomologists and forest pathologists should work together to build multidisciplinary curricula to sensitise students to the need to consider forest risks in a holistic manner and to educate future managers in integrated forest protection.
- Public plant health services could work with plant pathologists and entomologists to create early warning systems using citizen science to involve the public in tree health issues, including opportunities for learning and participation in scientific research, monitoring and surveillance.
- Entomologists and plant pathologists stand ready to assist decision- and policy-makers and forest managers in building global databases related to biological invasions, which will comprise information about threats, latest data on ongoing invasions, protocols and methodologies for eradication of emerging pests and pathogens, vectors of invasion and best practices for prevention.

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Authors' contributions

HJ, MLDL, AB, EGB, AS and JS designed the opinion paper and wrote the first draft, with subsequent contributions by all other authors.

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The potential current distribution of the coypu (*Myocastor coypus*) in Europe and climate change induced shifts in the near future

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Abstract

The coypu (*Myocastor coypus*) is a semi-aquatic rodent native to South America which has become invasive in Europe and other parts of the world. Although recently listed as species of European Union concern in the EU Invasive Alien Species Regulation, an analysis of the current European occurrence and of its potential current and future distribution was missing yet. We collected 24,232 coypu records (corresponding to 25,534 grid cells at 5 × 5 km) between 1980 and 2018 from a range of sources and 28 European countries and analysed them spatiotemporally, categorising them into persistence levels. Using logistic regression, we constructed consensus predictions across all persistence levels to depict the potential current distribution of the coypu in Europe and its change under four different climate scenarios for 2041–2060. From all presence grid cells, 45.5% showed at least early signs of establishment (records temporally covering a minimum of one generation length, i.e. 5 years), whereas 9.8% were considered as containing established populations (i.e. three generation lengths of continuous coverage). The mean temperature of the warmest quarter (bio10), mean diurnal temperature range (bio2) and the minimum temperature of the coldest month (bio6) were the most important of the analysed predictors. In total, 42.9% of the study area are classified as suitable under current climatic conditions, of which 72.6% are to current knowledge yet unoccupied; therefore, we show that the coypu has, by far, not yet reached all potentially suitable regions

in Europe. Those cover most of temperate Europe (Atlantic, Continental and Pannonian biogeographic region), as well as the coastal regions of the Mediterranean and the Black Sea. A comparison of the suitable and occupied areas showed that none of the affected countries has reached saturation by now. Under climate change scenarios, suitable areas will slightly shift towards Northern regions, while a general decrease in suitability is predicted for Southern and Central Europe (overall decrease of suitable areas 2–8% depending on the scenario). Nevertheless, most regions that are currently suitable for coypus are likely to be so in the future. We highlight the need to further investigate upper temperature limits in order to properly interpret future climatic suitability for the coypu in Southern Europe. Based on our results, we identify regions that are most at risk for future invasions and provide management recommendations. We hope that this study will help to improve the allocation of efforts for future coypu research and contribute to harmonised management, which is essential to reduce negative impacts of the coypu and to prevent further spread in Europe.

Keywords

biological invasions, climate change, consensus prediction, invasive alien species management, nutria, species distribution modelling, vertebrate

Introduction

Invasive alien species, i.e. species introduced to areas outside their native range that have become successfully established, spread and cause substantial impacts on the new environment (CBD 2002), are one of the main constituents of global change (Simberloff et al. 2013). They are a major cause of biodiversity loss, often associated with significant economic losses and negative impacts on human health (IPBES 2019).

One prominent example, even included in the list of “100 of the World’s Worst Invasive Alien Species” (Lowe et al. 2004), is the coypu, *Myocastor coypus* (Molinia 1782). This large semi-aquatic rodent native to subtropical and temperate South America was introduced to many regions of the world and subsequently often became invasive in those regions of introduction, for example, in Europe, North America and Asia (Carter and Leonard 2002, Hong et al. 2015, Ojeda et al. 2017, Tsiamis et al. 2017, Kawamura et al. 2018). The fur industry, being the main historic invasion vector beside zoos, game and biocontrol (e.g. for removal of aquatic vegetation), has led to the establishment of coypu farms all over the world, with the first registered introductions to Europe dating back to the second half of the 19th century (Carter and Leonard 2002, Scheide 2013, Tsiamis et al. 2017). Escaped or intentionally-released animals, as well as deliberate introductions subsequently served as source for wild populations (Carter and Leonard 2002, Tsiamis et al. 2017).

Negative impacts of the coypu are mainly due to its burrowing activity and feeding behaviour and include undermining of flood protection structures, such as river banks and dykes and therefore increased risk of floods, as well as agricultural damage, mainly on corn and sugar beet (Gosling and Baker 1989, Woods et al. 1992, Carter and Leonard 2002, DAISIE 2009, Scheide 2013, Tsiamis et al. 2017). For instance, within a six year period in Italy, damage amounted to about €1 million in agriculture and more than €10 million were attributed to the destruction of riverbanks (Panzacchi et al. 2007). Vari-

ous studies report that dense coypu populations can reduce plant diversity and destroy seedlings, influencing vegetation succession and preventing re-vegetation in marshes and wetlands. There are reports of coypus severely affecting wetland vegetation, for example, in Italy (Bertolino et al. 2005, Prigioni et al. 2005), Great Britain (Gosling and Baker 1989) and Louisiana (Baroch et al. 2002). Additionally, the coypu is a potential vector of hazardous diseases such as leptospirosis, toxoplasmosis and trichinosis (Carter and Leonard 2002, Scheide 2013, Fratini et al. 2015). Another aspect is the potential negative impact on breeding success of marshland birds by using floating nests as resting ground and consequently destroying or sinking the eggs (Bertolino et al. 2012).

The coypu is an opportunistic herbivore, preferably inhabiting slow-flowing or standing water bodies that are rich in hydrophytes, reeds and riparian vegetation, as well as wetland areas and swamps in lowlands (Woods et al. 1992). According to Baroch et al. (2002), coypus are capable of long distance dispersal, although they usually do show philopatric behaviour. However, if the environmental conditions become suboptimal, for example, due to drought or limited food resources, coypus may migrate. In this case, they primarily disperse along waterways (Hong et al. 2015). Although there is little information on coypu dispersal behaviour in general, there are reports of a range expansion in Eastern Europe of up to 120 km within a two-year period (Aliev 1968), dispersal distances of 67 km within eight years along the Norfolk river (Gosling and Baker 1989) and about 50 km per year at the lower Nakdong River in South Korea (Hong et al. 2015). In contrast, coypus barely travel more than 200 m away from aquatic habitats while foraging (Scheide 2013) and Denena et al. (2003) showed very small individual daily linear travel distances (143–475 m) under favourable environmental conditions. Coypus are non-seasonal breeders and have a high reproduction rate with about 2 to 3 litters per year (litter size 1–12) under favourable conditions (Woods et al. 1992, DVWK 1997, Guichón et al. 2003, Scheide 2013). Mild winters favour rapid population growth, through decreased fitness loss and mortality, as well as additional reproduction events in the cold period (Gosling and Baker 1989, Woods et al. 1992), underlining the importance of considering recent climate change in studies on this species.

Nowadays, the coypu is established in many European regions (Tsiamis et al. 2017). However, management measures differ across Europe, often even within countries. In some countries, the coypu is included in hunting laws or hunting is allowed with exceptional permission. Others conduct intensive control programmes with government trappers and volunteers such as farmers, for example, in Belgium (Verbeylen 2002, K Swinnen personal communication), France (Carter and Leonard 2002), Italy (Bertolino, Perrone & Gola 2005; S Bertolino personal communication) and the Netherlands (Unie van Waterschappen 2017; D Moerkens personal communication). In Great Britain, a successful eradication programme was undertaken between 1981 and 1989 (Gosling and Baker 1989). Recently, the coypu became listed as one of 66 invasive alien species of Union concern (EU Commission 2016, 2017, 2019) associated with the EU regulation on invasive alien species (EU Commission 2014). Member states are therefore obliged to implement strategies, which encompass the prevention of introduction and spread, early detection and eradication or management of those species.

However, successful management requires an adequate understanding of the ecology and behaviour of the targeted species (Jiménez-Valverde et al. 2011, Kawamura et al. 2018). In particular, assessing current and future potential distribution, taking into account global change, is key for successful management and the identification of priority management and monitoring regions (van Klinken et al. 2015). One widely used approach to reveal potential distributions of invasive alien species is via species distribution models (SDMs), which correlate a species' occurrence in geographical space with environmental variables in order to predict its potential distribution through spatial and temporal extrapolation (Elith and Leathwick 2009, Václavík and Meentemeyer 2009, Franklin 2010, Jiménez-Valverde et al. 2011). Although some studies have applied SDMs to the coypu (Bertolino and Ingegno 2009, Scheide 2013, Farashi and Najafabadi 2015, Hong et al. 2015, Jarnevich et al. 2017), a detailed investigation on a pan-European scale, taking into account land cover, bioclimatic and socioeconomic factors, is yet missing.

In the light of the urgent need of a harmonised coypu management, here we provide such an assessment for Europe. Specifically, we reconstruct the recent spread within the last decades and the current distribution of the coypu and group the occurrence data into different persistence-categories to identify regions that are suitable for permanent occurrence of coypus. Further, by using a consensus approach, we predict its potential current distribution and analyse to what extent suitable regions are not yet invaded. Finally, we investigate which climatic, land cover and socioeconomic variables influence coypu occurrence and model the potential future distribution under four different climate change scenarios. Based on our results, we identify regions that are most at risk for future invasions and provide management recommendations.

Methods

Study area and data acquisition

The study region includes most parts of the European mainland and the larger islands (excluding only European Russia, Ukraine, Belarus and Cyprus) (Fig. 1). To provide an up-to-date overview of the current distribution of the coypu, data from several sources such as publications, national administrative authorities and scientists were compiled between July 2017 and December 2018 (Suppl. material 1, Table S1). Additionally, we downloaded occurrence data from the Global Biodiversity Information Facility (GBIF.org). The occurrence of persisting populations in the Boreal, Arctic and northern Alpine biogeographic regions (European Environment Agency 2002) is not mentioned in literature (Carter and Leonard 2002, DAISIE 2009, Tsiamis et al. 2017), thus, no further effort has been made in sending out data requests for those regions. The same applies to Portugal, from where occurrences are not known either (Carter and Leonard 2002, Tsiamis et al. 2017). We did not include data from the coypu's native range, because there are only few spatially explicit records available, as already pointed out by Jarnevich et al. (2017).

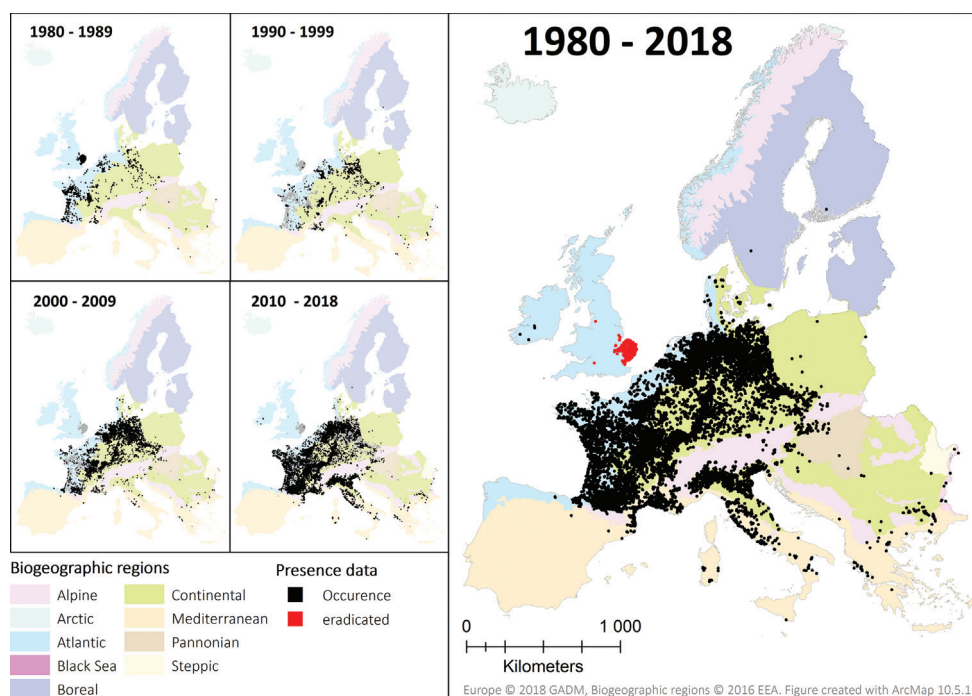


Figure 1. Coypu occurrence records from 1980 to 2018 in Europe. The decade-wise accumulation of records is depicted on the left side, with records of the respective decade in black and records of previous decades shown in grey. On the summary map (right side), records in Great Britain are displayed in red, as the coypu is officially eradicated (see Gosling and Baker 1989). Note, that for optimised illustration purposes, 10-km buffered centroids of occurrence records are shown.

Occurrence data

The raw occurrence data were prepared and quality-checked prior to analyses. If a source described the occurrence of coypu over several decades, the record was split into one record per decade. Only records that contained geographic information and approximate sampling date were considered in this study. Records that were lacking coordinates, but contained an unambiguous locality description, were georeferenced, either using the point-radius method (estimating coordinates and an uncertainty radius according to the precision of the locality description) or the shape method (assigning a geographic shape that represents the uncertainty) (Wieczorek et al. 2004); i.e. for France and Germany, locality descriptions at municipality levels were linked to the according feature of the GADM 3.4 shapefile (database of Global Administrative Areas, <https://gadm.org/>). Point-radius georeferencing was conducted using the GeoLocate web tool (Rios and Bart 2010) and web map services. Coordinate uncertainty estimates were used to buffer the records. Note that for records linked with the municipality area, no buffers were introduced; thus, the uncertainty information was linked with the shape and size of the municipalities. As the coypu is a mobile species, a circular buffer of 1 km radius

was applied to records with very low uncertainty estimates to sufficiently cover potential home ranges and account for cases where records fall at the borders of grid cells. After a literature research on coypu home ranges (Doncaster and Micol 1989, Denena et al. 2003, Nolfo-Clements 2009, Scheide 2013), we have orientated ourselves to the upper end of documented values. Scheide (2013) reported that, along waterways, territory length usually varies between around 150 m to about 1 km, although home range can increase up to several kilometres in radius if resources are scarce.

For further analysis, records that were missing essential information (i.e. no geo-reference), putative duplicates (i.e. records with same year and coordinates or locality description), as well as records exceeding an uncertainty radius of 10 km in areas where more accurate records were available, were discarded. This resulted in a final dataset consisting of 24,232 coypu records between 1980 and 2018 across 28 European countries containing year, uncertainty estimate and coordinates (Fig. 1).

We transformed those presence records (inclusive uncertainty buffer) to a grid of 5×5 km resolution (temporal resolution: one year) to reduce the effect of pseudo-replication (i.e. artificial inflation of the sample size due to intensively-sampled regions or non-detected duplicates in different datasets). Grid cells that showed only marginal overlap with buffered presences ($< 2.5\%$ of the grid cell area) were not defined as presences, to avoid area inflation. After discarding those, 25,534 grid cells (about 12.6% of all grid cells throughout the study area) were defined as presence grid cells and were used for further analysis (see workflow scheme, Fig. 2). Note, that there are more presence grid cells as presence records per se, as we used the buffered presences for the grid cell transformation to consider the spatial uncertainty of records.

Spatiotemporal analysis

Dullinger et al. (2009) found that environmental niche models, based on distribution data, produce more accurate predictions when analyses are restricted to persistent populations. To keep information about long term occurrence and, therefore, probable establishment, we conducted a spatiotemporal analysis and allocated the presence within grid cells to different persistence categories. Presence records per grid cell were analysed by counting the number of years containing coypu records and the time-span covered. Grid cells with multiple years of recorded coypu presence were classified with regard to the time-span covered by the records and accordingly grouped into different persistence levels of at least one, at least two or at least three generation lengths (hereafter GL; which is 5 years, according to Ojeda et al. (2017)). Temporal coverage of at least one or two generation lengths can be interpreted as early signs of establishment. For cells that repeatedly showed coypu presence, covering a time-span of at least 15 years (thus three full generation lengths of coypu), we assumed the occurrence of established populations within the time from 1980 to 2018. If temporal discontinuities of more than 10 years between successive records were present in a grid cell, we divided the counts into subgroups, which were analysed separately. In this case, the highest derived persistence category was assigned to the according grid cell.

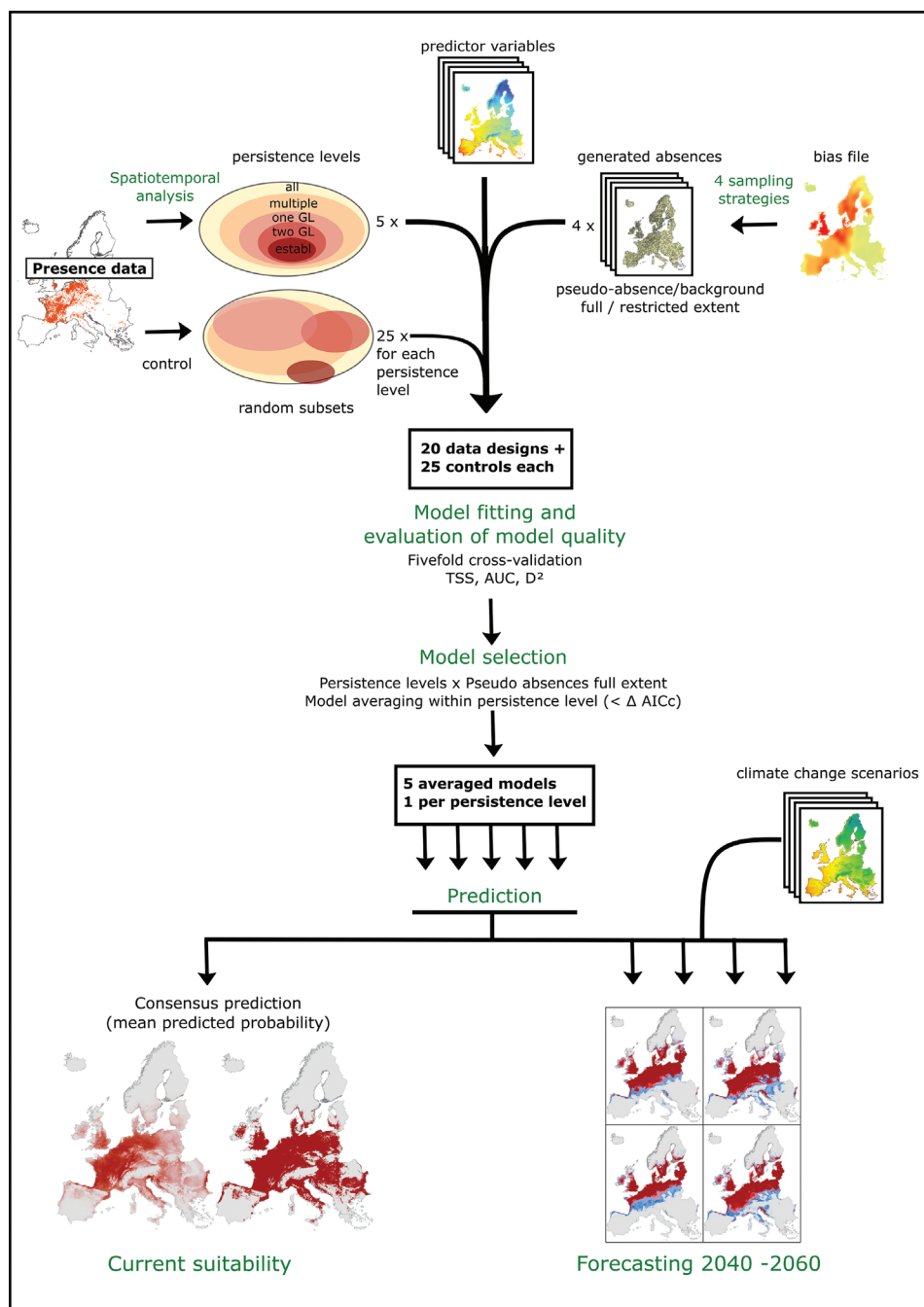


Figure 2. Workflow used for species distribution modelling. Presence data was transformed to a raster grid (5×5 km), spatiotemporally analysed, accordingly grouped into five levels of persistence and then combined with environmental data and generated absences (using four different sampling strategies) to fit logistic regression models. The absence sampling strategy that derived the best evaluation measures was used for predicting suitability under current (1979–2013) and future climatic conditions after going through a model selection and averaging procedure.

On this basis, we created sub-datasets by stepwise exclusion of the lowest level of persistence (those sub-datasets are hereafter called persistence levels and abbreviated as indicated by the bold words: **all** grid cells = 25,534, **multiple** records per grid cell = 15,078, multiple records per grid cell that cover at least **one GL** = 11,619, multiple records per grid cell that cover at least **two GL** = 5,145, **established** populations only = 2,505).

Predictor variables

Environmental predictors included bioclimatic variables from the CHELSA database (Karger et al. 2017a), CORINE Land Cover data for different land cover classes (i.e. agricultural areas, wetlands and water bodies) (European Environment Agency (2012), hilliness (i.e. the standard deviation of the average sea level) derived from a digital elevation model (European Environment Agency 2000), human population density (EUROSTAT 2011) and data on rivers and lakes (European Environment Agency 2012b) (Table 1). Human population density was log-transformed to reduce the effect of outliers due to large cities. All variables were calculated for a grid of 5 × 5 km and standardised for better comparison (i.e. scaled to a mean of 0 and a standard deviation of 1). As multiple bioclimatic variables were highly correlated ($| \text{Pearson's } r | > 0.7$), we limited them to five variables that represent temperature (bio06; ‘Minimum Temperature of Coldest Month’ and bio10; ‘Mean Temperature of Warmest Quarter’), temperature fluctuations (bio2; ‘Mean Diurnal Range’), precipitation (bio17; ‘Precipitation of Driest Quarter’) and precipitation fluctuations (bio15; ‘Precipitation seasonality’). After this procedure, all pairwise correlations of our final predictor set were below the threshold of $| \text{Pearson's } r | > 0.7$ and all variables showed VIF (Variance Inflation Factor) values smaller than 10, indicating that collinearity is a minor issue within the predictor data (R package usdm, Naimi et al. 2014) (for correlations amongst variables, see Suppl. material 1, Fig. S1).

Additionally, we used qualitative information on biogeographic region (European Environment Agency 2016) and country (GADM 2018) for summary statistics.

Table 1. Environmental predictor variables. All predictors were rescaled to a 5 × 5 km raster resolution (bilinear interpolation) and standardised (scaled to a mean of 0 and a standard deviation of 1).

Predictor	Description	Temporal coverage	Source
Bio2	Mean Diurnal Range [°C]	1979–2013	CHELSA (Karger et al. 2017)
Bio6	Min Temperature of Coldest Month [°C*10]		
Bio10	Mean Temperature of Warmest Quarter [°C*10]		
Bio15	Precipitation Seasonality [CV]		
Bio17	Precipitation of Driest Quarter [mm/quarter]		
Hilliness	Std. Dev. of m a.s.l./cell	2000	EEA
Pop. density	Mean human population density [inhabitants/km ²] log-transformed	2011	GEOSTAT v.2.0.1. / Eurostat, EFGS
Distance Settlement	Euclidean distance to the next grid cell containing artificial surfaces	2012	CORINE LANDCOVER, vers. 18.5.1
Agriculture	Agricultural surfaces [% counts/cell]		
Wetlands	Wetlands surfaces [% counts/cell]		
Waterbodies	Water bodies’ surfaces [% counts/cell]		
Shores	Total shoreline (rivers and lakes) [m/ha]	1990–2006	ECRINS v.1.1 / EEA

Future climate projections

Two IPCC (Intergovernmental Panel on Climate Change) climate change scenarios were selected to model coypu response to a changing climate by the mid-21st century (2041–2060). One represents medium (RCP 4.5; Representative Concentration Pathway) and one represents severe climate change (RCP 8.5) by depicting the different approximate radiative forcing in comparison to the pre-industrial state (i.e. + 4.5 and + 8.5 W/m²) (Moss et al. 2010). Further, it is known that climate predictions are sensitive to different climate model frameworks (GCMs; General Circulation Models) (Porfirio et al. 2014). Thus, we downloaded data for two different GCMs, representative for different model families (Sanderson et al. 2015), from the CHELSA website (Karger et al. 2017a). The GCMs were chosen considering model independence and performance: Had-GEM2-A0 and CESM1-BGC.

Presence-absence data designs

To model the range of potential distribution under current climate and under climate change, we used logistic regression, a generalised linear modelling (GLM) technique that is widely used for predicting species distributions (Elith and Leathwick 2009, Franklin 2010). Here, a linear model is related to the binary response variable via a logistic link function (McCullagh and Nelder 1989). We chose this technique due to the proposed transferability in time and space and lower risk of overfitting compared to other methods, such as classification trees (Marmion et al. 2009, Franklin 2010). Since logistic regression requires presence and absence data, we generated absences using different approaches to compare their model performance and predictions in order to select the most appropriate absence design. The absences were drawn either as background or pseudo-absences, i.e. from all grid cells or only from non-presence grid cells (see Phillips et al. 2009); this was done for the whole study area extent or within a buffer of 150 km around presence grids, representing an assumption of coypu dispersal (Aliev 1968, Gosling and Baker 1989, Hong et al. 2015). As the data collected consists of a variety of sources, contains opportunistic records and is not following a standardised collecting scheme across our study area, we used a target-group approach to account for biased survey effort (Phillips et al. 2009, Stokland et al. 2011). Here, we filtered occurrence data of non-marine, non-volant, small- to medium-sized European mammals from GBIF (<https://www.gbif.org/>) for the same spatial and temporal extent and downloaded the resulting records, assuming these will exhibit a similar spatial sampling bias. The records were used to create a probability density surface (Suppl. material 1, Fig. S2a) that served as the basis for generating absences.

We combined each of the four different absence sampling strategies (background full, background restricted, pseudo-absence full, pseudo-absence restricted; see Suppl. material 1, Fig. S2b) with all five levels of persistence, resulting in a total of 20 data designs. The number of absence grids cells was set to at least 10,000 or equal the number

of presence grid cells. Barbet-Massin et al. (2012) argue that this amount of generated absences adequately depicts the model quality without the need to account for variability in generated absences, for example, through generating replicates. At the same time, predictive accuracy of GLMs does not significantly increase with prevalence, once the number of presences reached at least one tenth of the number of absences (Barbet-Massin et al. 2012). In their study, this held true for weighted and unweighted schemes, therefore we did not apply any weightings.

As pseudo-absences which were generated across the whole study area extent (“pseudo-absence full”) consistently derived the best evaluation values (Suppl. material 1, Fig. S2c) and spatial predictions were basically identical between different absence sampling strategies, we chose this sampling strategy for further analysis.

Model evaluation

We assessed the goodness of fit for the full models (including linear and quadratic terms for all variables) of all datasets and of 25 random subsets per persistence level. Those were created by drawing random presence subsamples that equal the size of the according persistence level to check for sampling size effects that might occur. For the model evaluation, we compared a set of commonly-used measures (Allouche et al. 2006, Elith and Leathwick 2009, Liu et al. 2011), based on a fivefold cross-validation (split ratio train:test equals 80:20): 1) AUC, which is the sum of the area under the receiver operating curve, a graph that displays false positive vs. true positive rate (Franklin 2010); 2) adjusted D^2 , which is the proportion of explained deviance,

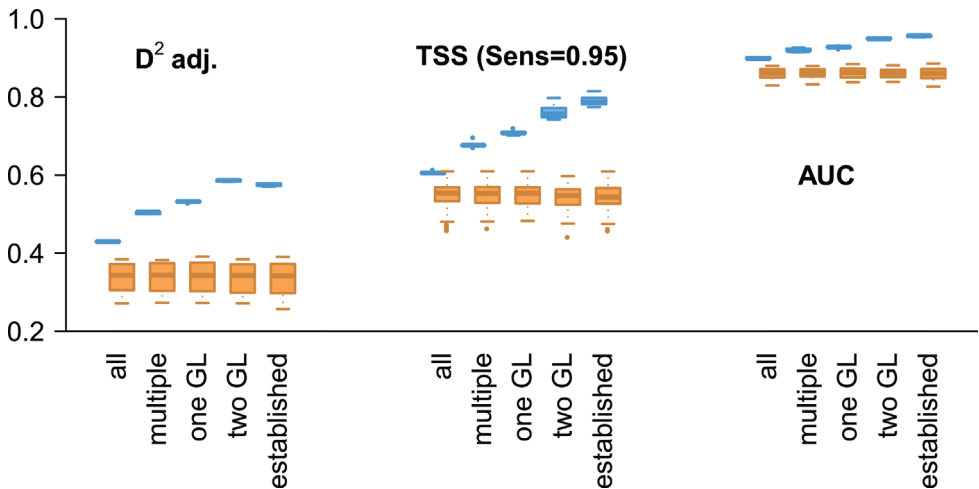


Figure 3. Comparison of model performance for the persistence levels (with pseudo-absences for the full extent) versus random subsets. The boxplots show the results of the fivefold-cross validation for the persistence levels (blue) and the according random subsets (orange).

taking into account the number of model parameters and observations and thus allows comparison amongst different models (R package *modEvA*; Barbosa et al. 2014); and 3) the threshold-dependent true skill statistic (TSS) which needs a binary result. TSS corresponds to the sum of sensitivity (i.e. the proportion of correctly-predicted presences) and specificity (proportion of correctly-predicted absences) minus one and was shown to be independent of prevalence (Allouche et al. 2006). As we aimed to identify the regions sensitive to coypu invasion and regarded false negatives as costlier than false positives, the threshold for TSS computation was chosen following the recommendation of Jiménez-Valverde et al. (2011) to avoid omission error by maximising sensitivity whilst keeping a reasonable specificity and therefore TSS value. Therefore, we set the sensitivity to a fixed value of 0.95 (i.e. the threshold used for binary classification will lead to 95% of presences predicted correctly).

Variable importance was measured for each predictor by evaluating the mean drop in explained deviance caused by removal of the respective predictor. Finally, true positives and omission errors were mapped to reveal sensitivity issues and spatial patterns in model performance (Suppl. material 1, Fig. S3).

Model selection and consensus predictions of potential current and future distribution

The full models' quality notably increased with increasing level of persistence and this effect could be clearly distinguished from sample size effects, when comparing the evaluation measures with those of the random subsets (Fig. 3). We hence used the persistence level classification for prediction. To select the best models for each persistence class, we compared the corrected Akaike's Information Criterion (AICc) between the full model (all predictors) and possible sub-models. Then we averaged the top models with $\Delta\text{AICc} < 4$ (R package "MuMIn", Barton 2019), finally resulting in five averaged models (one per persistence level) that were fitted with the whole data of the according persistence level.

We assumed grid cells that contain long-term occurrences to be more informative than those where coypu occurrence was only registered once. Both approaches (using all data vs. subsets) in its extremes may incorporate biases (i.e. all presence data will more likely include non-persistent occurrences and false identifications, whereas grid cells that show long-term occurrence might underestimate the area of a still-spreading alien species and comprise historical effects of propagule pressure due to regional differences in fur farming intensity, as well as effects of uneven data availability across regions). To balance those possible biases and reduce uncertainty, we combined the resulting predictions of probability of occurrence for all persistence levels and created a consensus prediction by simply calculating the mean probability of occurrence per grid cell, depicting the overall agreement of the averaged models. Marmion et al. (2009) showed a significant increase in accuracy and robustness for consensus predictions that used averaging methods. For binary maps, we used the

same cut-off as for the computation of the TSS (sensitivity fixed to 95%) to separate suitable and unsuitable grid cells (Jiménez-Valverde et al. 2011). The proportion of suitable grid cells was calculated for all countries and compared to the proportion of grid cells that contained presence records.

Further, consensus forecasts for all climate change scenarios were computed. The change in probability of occurrence was assessed by comparing the number of cells that were classified as suitable under current climate and under climate change scenarios and by subtracting the probabilities of occurrence of current from future predictions. To obtain the agreement between binary models, we used the sum of predicted presences per cell across all averaged models, with a high value meaning high agreement.

Priority regions for surveillance and management

Finally, we used the resulting predictions to define priority regions for surveillance and management by creating a risk map. Grid cells that 1) show high probability of occurrence in the consensus prediction and 2) areas adjacent to already known recent occurrences, are deemed to be particularly susceptible to invasion by coypus, due to short colonisation distances. Thus, to incorporate dispersal constraints and to account for proximity to known occurrence, a weighting matrix was computed, by summing up weighted inverse Euclidean distance classes per decade for each cell (Suppl. material 1, Fig. S4). We combined this matrix with the consensus map under current climate showing the mean probability of occurrence. Here, values below the probability threshold for binary map computation were set to zero. We expected both suitability and proximity to be of equal importance for the invasion process. If a cell were considered too distant or unsuitable, no risk of invasion was assumed. For the United Kingdom, we considered only Northern Ireland for the risk map and excluded Great Britain, as the coypu has been eradicated there (see Gosling & Baker 1989).

Statistical analyses were conducted and maps were produced using ArcGIS 10.5.1 (ESRI 2018), R 3.6.0 (R Core Team 2019) within the GUI RStudio 1.1.463 (RStudio Team 2018). The following R packages were used: *dismo* (Hijmans et al. 2017), *dplyr* (Wickham et al. 2017), *modEva* (Barbosa et al. 2016), *MuMIn* (Barton 2019), *Presence-Absence* (Freeman and Moisen 2008), *raster* (Hijmans 2017), *spdep* (Bivand et al. 2013, Bivand and Piras 2015), *rgbif* (Chamberlain et al. 2017), *usdm* (Naimi et al. 2014).

Results

Spatiotemporal analysis of coypu occurrence

In total, 24,232 coypu presence records (corresponding to 25,534 grid cells at 5 × 5 km) were collected across 28 countries. The spatiotemporal analysis of presence grid cells shows centres of documented long-term occurrence in Czech Republic, France,

Germany, Italy and the Netherlands. Of all presence grid cells, 45.5% (corresponding to 20 countries) show at least early signs of establishment (i.e. had multiple records that covered one generation length as a minimum; of those 20.1% have been covered by at least two generation lengths and 9.8% of the grid cells (corresponding to 10 countries) show spatially-explicit evidence for long-term persistence (i.e. established populations) with coypus being present over a period of at least 15 years (Fig. 4). Note, that these periods of occurrence do not necessarily imply that populations are still present in a given area, but are indicative of the general suitability of the area within the last decades. For example, the successful eradication programme in Great Britain led to the local extinction of the coypu (Gosling and Baker 1989), despite the suitability of the environment that allowed persistence over several generation lengths.

Model performance and variable importance

Model quality increased with higher levels of persistence, with mean AUC values ranging from 0.90 (all) to 0.96 (established) indicating excellent discrimination ability across all averaged models and TSS values ranging from 0.61 to 0.79 which can be interpreted as good to excellent agreement between training and test data (Eskildsen et al. 2013). Adjusted explained deviance was between 43.0% and 57.5% (Table 2).

Between two to four top models were averaged for the persistence levels, with the full model always being included. Only land cover variables were excluded ('shores', 'water-bodies') and none of them was excluded across all persistence levels (for model weightings and ΔAIC_c , see Suppl. material 1, Table S2). The analysis of predictor variable importance showed that the mean temperature of the warmest quarter (bio10; mean drop in $D^2 \pm SD$: 8.5 ± 0.77), mean diurnal temperature range (bio2; 7.8 ± 3.51) and the minimum temperature of the coldest month (bio6; 3.2 ± 0.22) were the most important of the analysed predictors (Fig. 5). In addition, precipitation seasonality (bio15; 2.1 ± 0.41) played a relatively important role, whereas the other variables had markedly lower values. Still, the mean precipitation of the driest quarter (bio17), hilliness and the distance to settlements are of superior importance in comparison to the land cover variables. Coypu presence was more likely at medium diurnal temperature ranges and when the mean temperature of the warmest quarter, as well as the minimum temperature of the coldest month, was medium to high. The probability of occurrence decreased with increasing precipitation seasonality, distance to settlements and hilliness and increased with increasing human population density, number of wetlands and higher precipitation during drier months (bio17).

Plotting of the omission errors of the binary consensus prediction revealed that those mostly occurred at the range margins of the currently known European distribution, especially towards Southern Europe and mountainous areas (Suppl. material 1, Fig. S3b). A good distinction between the presence and generated pseudo-absence data could be achieved (median of predicted probability for pseudo-absences and presences: 0.05 vs. 0.77, Figure S3a).

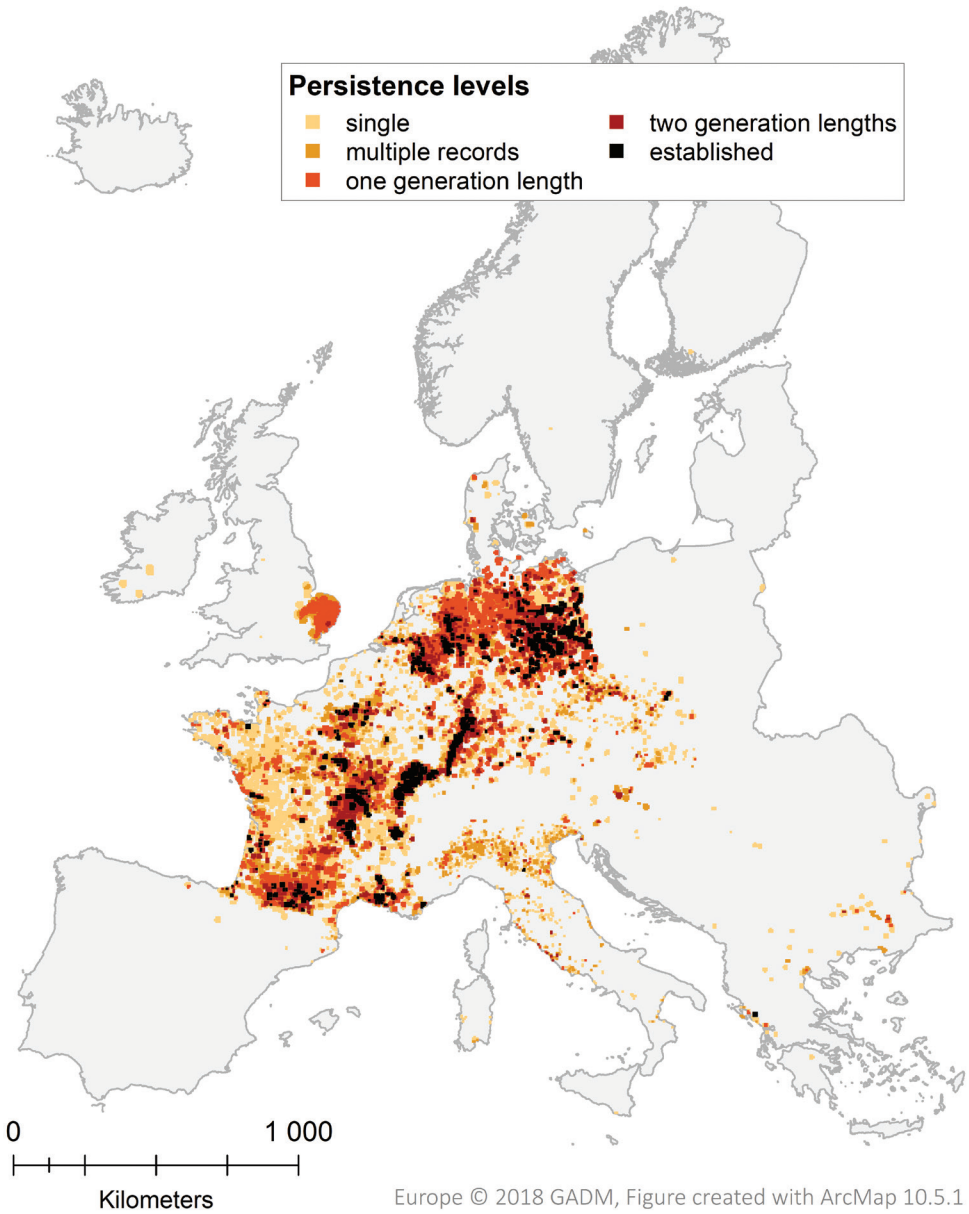
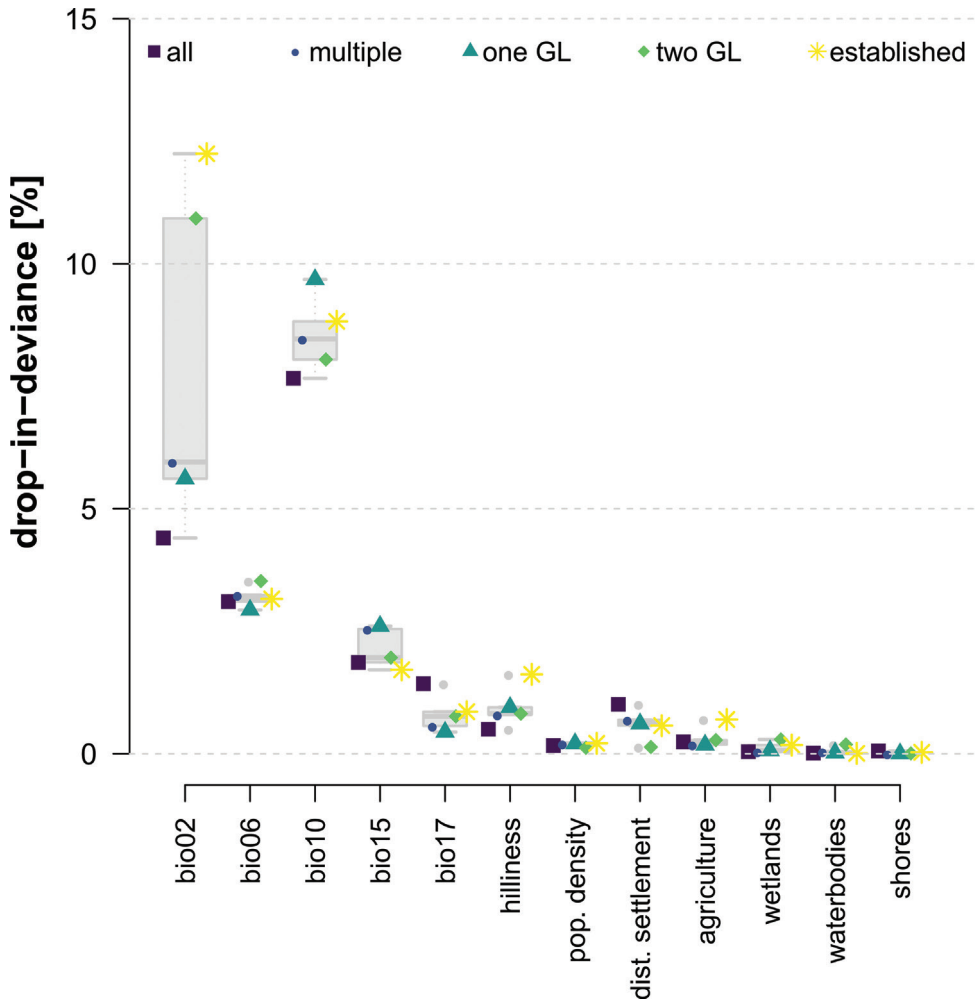


Figure 4. Persistence levels of presence grid cells as derived from the spatiotemporal analysis. Each grid cell that intersects at least one record of coypu presence between 1980 and 2018 is coloured according to the maximum derived persistence level: 1) single record, 2) multiple records, 3) one generation length (multiple records covering at least 5 years), 4) two generation lengths (multiple records covering at least 10 years), 5) established (multiple records covering at least 15 years). One generation length is assumed to be 5 years, following Ojeda et al. (2016).

Table 2. Evaluation statistics of the averaged models for all five levels of persistence. For computation of the TSS, the sensitivity was set to 0.95.

	AUC	D ² adj [%]	TSS (Sens = 0.95)	Specificity
all	0.90	43.0	0.61	0.65
multiple	0.92	50.3	0.68	0.73
one GL	0.93	53.1	0.71	0.76
two GL	0.95	58.6	0.76	0.81
established	0.96	57.5	0.79	0.84

**Figure 5.** Importance of predictor variables for the averaged final models. The importance is measured as the mean drop in explained deviance (D^2) upon removal of the respective predictor. For descriptions of the predictor variables, see Table 1.

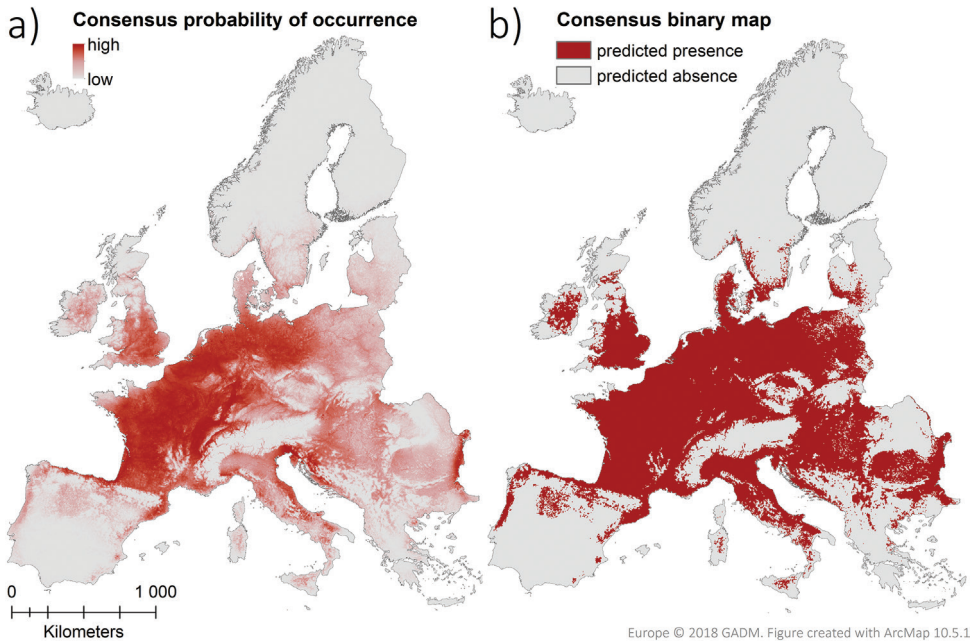


Figure 6. Consensus predictions of the probability of occurrence across the study area under current climatic conditions (years 1979–2013). **a** Mean probability of occurrence across the final averaged models of all persistence levels. **b** Binary classification of suitable and unsuitable grid cells after applying a threshold corresponding to 0.95 sensitivity (= 0.16).

Current and future predictions of potential distribution

The consensus map for current climatic conditions shows that, currently, large parts of Europe have a high probability of coypu occurrence (Fig. 6a). Applying a threshold that gives 95% sensitivity (correctly predicted presence grid cells) results in 42.9% of the study area being rated as potentially suitable (Fig. 6b). Only 27.4% of those cells already comprise documented coypu occurrences, while the remaining 72.6% being, to our current knowledge, yet unoccupied. These potentially suitable areas cover most of Central Europe and parts of the following biogeographical regions; Atlantic (67.8%), Black Sea (92.7%), Continental (79.3%), Mediterranean (24.0%), Pannonian (93.5%) and Steppic (38.2%) regions. Only minor parts of the Alpine and Boreal regions contain predicted suitable grid cells (8.8% and 4.2%) and the Arctic biogeographic region is considered unsuitable.

All four climate change scenarios show substantial shifts in predicted habitat suitability until the mid-21st century (2041–2060) (Fig. 7). While the total amount of suitable area is predicted to decrease between 2–8% in comparison to current climatic conditions (38.1% (HadGEM1 A0 RCP 4.5), 34.7% (HadGEM1-A0 RCP 8.5),

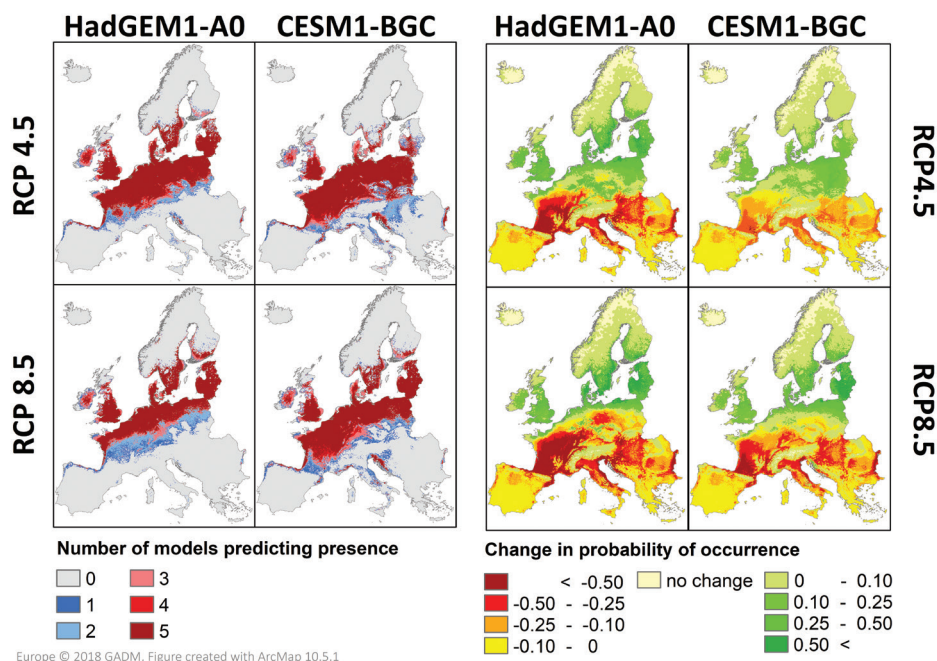


Figure 7. Future predictions. Agreement between averaged models for projected probability of occurrence in the mid-21st century under two climate change scenarios (medium climate change: RCP 4.5; severe climate change: RCP 8.5) combined with two different global circulation models (HadGEM1-A0, CESM1-BGC, displayed as number of models predicting presence (left side) and net change in occurrence probability compared to the current climatic situation (right side).

40.9% (CESM1-BGC RCP 4.5) and 39.8% (CESM1-BGC RCP 8.5), particularly northern and Atlantic regions with Ireland and the United Kingdom will experience an increase in suitability. In contrast, all models predict decreasing suitability along the southern range.

Priority regions for surveillance and management

The risk map (Fig. 8) is an indication of invasion risk, considering not only the potential suitability, but also the current distribution of the coypu and thus the likelihood of dispersal and colonisation of new grid cells. In Figure 9, the percentage of suitable and occupied area per country, as well as the establishment status of the coypu in the respective country, is shown. None of the already affected European countries reached saturation by now and, additionally, a number of not-yet invaded countries contain a considerable amount of suitable area.

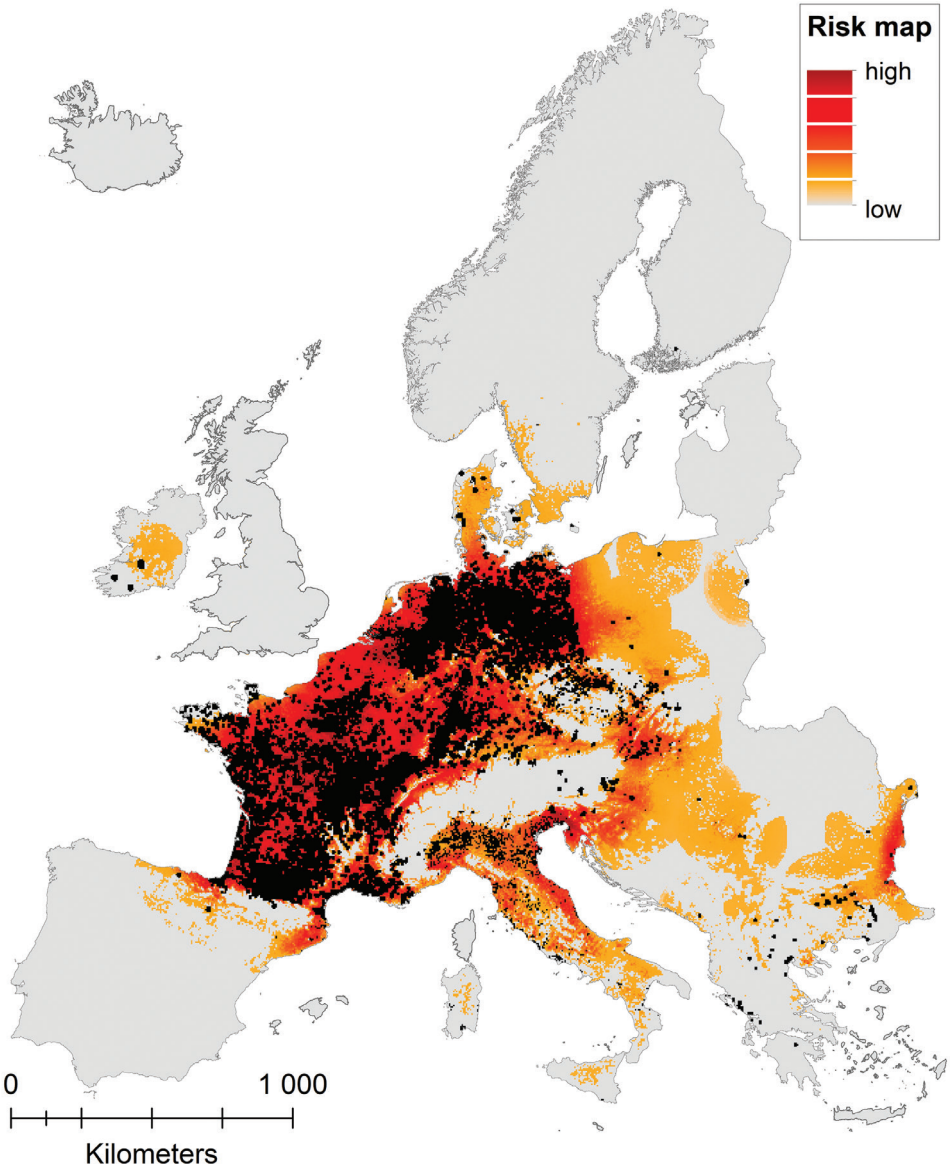


Figure 8. Risk map, highlighting regions potentially prone to invasion, i.e. with high probability of occurrence under current climate and adjacent to known recent occurrences of coypu. Presence grid cells are shown in black. Great Britain was excluded as the coypu is officially eradicated (Gosling & Baker, 1989).

Discussion

Current situation and changes in the near future

This study confirms and substantially expands the overview of Tsiamis et al. (2017), who report coypu occurrence for 18 EU countries and establishment for 12 of those. Our presence dataset covers 28 European countries, of which 10 countries showed spatially-explicit evidence for long-term establishment of the species and another 10 countries showed at least early signs of establishment within the regarded time period (Fig. 9; note that our study also deals with non-EU member states). Under the current climate, a considerable number of countries has high proportions of suitable areas, for example, Belgium, Germany, Luxembourg, Netherlands and Hungary with > 90% and Bulgaria, Croatia, Czech Republic, Denmark, France, Kosovo, Poland, Italy, Serbia, Slovenia, Slovakia and the United Kingdom with more than half of their country area being predicted as suitable for coypu (Fig. 9). The comparison of presence grid cells with not-yet invaded but suitable ones, shows that further substantial range expansions can be expected. Moreover, several countries that do not have documented coypu occurrences yet, contain potentially suitable areas. Overall, 42.9% of the study area is considered suitable under current climate (1979–2013) (Fig. 6), of which less than a third already contained occurrences from 1980 to 2018.

All four climate change scenarios used in this study predicted a slight to moderate decrease of suitable area (from 42.9% under current climate to between 34.7% and 40.9%). This decline is caused by a loss of suitable habitats in the southern parts of Europe, which is not fully compensated for by increasing suitability at higher latitudes (Fig. 7). Thus, our results show that climate change likely will not cause an overall increase of suitable areas for coypu in Europe. This is in line with Bellard et al. (2018) who reviewed modelling studies of climate change effects on alien species distributions and found that climate change will more frequently contribute to a decrease in alien vertebrate species range size. Currently suitable areas closely match warm temperate climates (fully humid or summer dry and with warm summers) after the Köppen-Geiger Climate Classification (Kottek et al. 2006), but not regions with hot summers. Scheide (2013) mentions increasing mortality rates of coypu at high temperatures, which would be in accordance with our finding of decreasing suitability in warming arid regions. In addition, Jarnevich et al. (2017) found support for upper thermal tolerance thresholds of the species. Decreasing suitability on the Iberian peninsula supports the findings of Gallardo and Capdevila (2018) which conducted a risk analysis for Spanish national parks using climate scenarios for 2050 and 2070; they predicted slight to medium decrease of suitability for the coypu in the majority of cases.

The recent occurrence of the coypu in Ireland caused the first Species Alert issued by a European Union Member State under the EU Regulation on Invasive Alien Species. Although those areas were only partly predicted correctly, a considerable area of Ireland is classified as suitable by our predictions. In line with our study, Scheide (2013) and Jarnevich et al. (2017) identified Ireland as having a high similarity

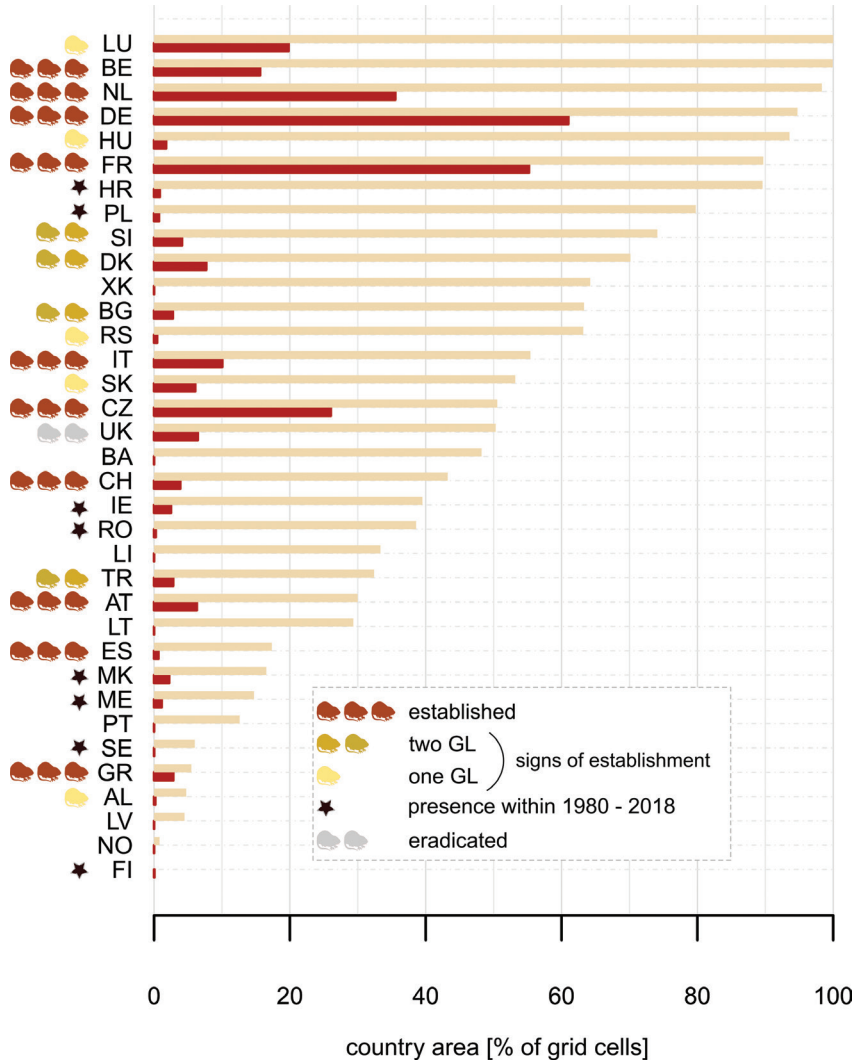


Figure 9. The country-wise percentage of suitable grid cells under current climate (brown bars) and grid cells containing presences (red bars). Countries are marked according to the maximum persistence level. Countries with no suitable areas and occurrences are not shown, as well as microstates not covering a whole grid cell. Alphabetically ordered country abbreviations with corresponding percentages of suitable grid cells and occupied cells in parentheses: AL : Albania (4.7/0.2), AT : Austria (30.0/6.3), BA : Bosnia and Herzegovina (48.2/-), BE : Belgium (99.9/15.6), BG : Bulgaria (63.3/2.8), CH : Switzerland (43.2/3.9), CZ : Czech Republic (50.5/26.0), DE : Germany (95.7/61.0), DK : Denmark (70.1/7.7), ES : Spain (17.3/0.6), FI : Finland (-/0.1), FR : France (89.7/55.2), GR : Greece (5.5/2.8), HR : Croatia (89.6/0.8), HU : Hungary (93.6/1.8), IE : Ireland (39.5/2.5), IT : Italy (55.4/10.0), LI : Liechtenstein (33.3/-), LT : Lithuania (29.3/-), LU : Luxembourg (100/19.8), LV : Latvia (4.5/-), ME : Montenegro (14.7/1.1), MK : Macedonia (16.5/2.2), NL : Netherlands (98.3/35.5), NO : Norway (0.8/-), PL : Poland (79.8/0.7), PT : Portugal (12.6/-), RO : Romania (38.6/0.2), RS : Serbia (63.2/0.5), SE : Sweden (6.0/<0.1), SI : Slovenia (74.1/4.1), SK : Slovakia (53.2/6.1), TR : Turkey* (32.4/2.8), UK : United Kingdom (50.3/6.4), XK : Kosovo (64.2/-).*) only the area of the European part of Turkey is considered.

to the coypu's realised niche when predicting its potential distribution on a global scale. Therefore, the recent occurrence reports should be taken with great caution, especially as the overall suitability of Ireland under climate change scenarios is expected to increase (Fig. 7). This said, the predictions of Jarnevič et al. (2017), when modelling the potential distribution of the coypu in the US and worldwide, did also classify large parts of Central and Eastern Europe as unsuitable, which is in contrast with our findings and with the presence of established populations in many areas of those regions.

While our predictions classify most of the Atlantic, Continental, Black Sea and Pannonian biogeographic regions as suitable, this is not the case for the Alpine biogeographic region. Therefore, this study is in agreement with others that have classified the coypu as a typical lowland species and implies that mountain regions act as effective dispersal barriers on a regional scale (Woods et al. 1992, DVWK 1997, Bertolino and Ingegno 2009, Scheide 2013).

Environmental predictors shaping coypu occurrence

Our results highlight the importance of temperature-related climatic variables, such as the mean temperature of the warmest quarter, the mean diurnal temperature range and the minimum temperature of the coldest month as being essential in shaping habitat suitability for coypus (Fig. 5). Under climate change, increasing populations due to decreasing winter mortality seem to be possible and could have economic and environmental consequences in affected areas (Gosling and Baker 1989, Carter and Leonard 2002, Scheide 2013).

Currently, urban coypu populations, fed by humans and profiting from mild urban climate and, in some cases, the thermal pollution of rivers, clearly demonstrate the consequences of high reproduction rates coupled with lowered mortality and enhanced resource availability for population densities (Carter and Leonard 2002, Verbeylen 2002, Walther et al. 2011, Scheide 2013). Whereas Meyer (2005) found local adaptations of the coypu to urban areas, other studies revealed negative effects of settlements on coypu occurrence (Bertolino and Ingegno 2009). In our study, increasing distance to settlements had a negative effect on occurrence probability, whereas the human population density was slightly positively correlated throughout all models. These results indicate that human presence seems to favour coypu occurrence, as it can well adapt to urban waters and can take advantage of additional resources provided for feeding. However, recording bias may contribute to this result as there may be preferential recording in more densely populated regions. Nevertheless, because the attitude toward coypu varies between regions or countries (Carter and Leonard 2002), the association between humans and coypu occurrence may vary spatially and may also change over time. While in some regions the species is hunted (Carter and Leonard 2002, Bertolino and Ingegno 2009) and therefore presence might be more likely in remote areas, in others, regularly fed urban populations occur (e.g. in Germany (Scheide 2013), the

Czech Republic (M Anděra personal communication) or in Austria (A Schertler personal observation)). In contrast to former findings, nowadays the coypu is also common in Italian cities, where it is fed by people, highlighting the temporal dynamic of its relationship with humans (S Bertolino personal communication).

Although on a continental scale, climatic aspects are clearly of higher importance (Franklin 2010), some of the predictors associated with land cover consistently showed a significant influence on occurrence probability which, for example, increased with increasing amount of wetlands within a grid cell. The relevance of land cover variables for coypu occurrence was reported by previous studies (Scheide 2013, Farashi and Najafabadi 2015) and should definitely be taken into account in future studies that are conducted on a finer scale. Bertolino and Ingegno (2009) showed that coypu prefers rice paddies as habitats in Northern Italy. Specific agricultural areas do not only enhance food availability, but also potentially provide habitat, for example, through irrigation ditches. The flexibility to colonise a variety of habitats must be considered when predicting the potential future distribution of the coypu. Although arid environments in Southern Europe are predicted to be unsuitable in the near future (Fig. 7), wetlands and small patches of suitable habitat due to microclimatic factors (e.g. along riparian areas), as well as agroecosystems can still provide suitable habitat for coypus and simultaneously promote conflicts due to feeding damage.

Predictive ability of the species distribution model

The assumption of an equilibrium between a population and its environment is typically violated during biological invasions, due to ongoing dispersal. Václavík and Meentemeyer (2012) found that SDMs calibrated in early invasion stages tend to be less accurate and under-predict potential ranges of species. The coypu was introduced to Europe more than a century ago and a multitude of release or escape events across regions happened, as it was widely used for fur farming (Carter and Leonard 2002). As the coypu is a conspicuous species that is regularly recorded by a wide range of people (e.g. naturalists, waterway authorities, anglers, farmers and hunters) and given the exhaustive search of records performed, we are convinced that the collated distribution dataset closely reflects the known distribution of the coypu in Europe. Therefore, we assume that our dataset captures a wide range of suitable environmental conditions, with the consensus predictions providing valuable tools to predict the next phase of invasion and areas at high risk (Fig. 8).

The SDMs performed well (Table 2), although some tendency in misclassifications was detected (Suppl. material 1, Fig. S3b). This might be due to missing essential predictors, model mis-specification or influential spatially clustered factors, such as biotic interactions, propagule pressure and dispersal (Elith and Leathwick 2009). Propagule pressure and the number of release events within the last century are difficult to reconstruct given the lack of necessary data, but were presumably differing across Eu-

rope, due to the varying economic importance of the fur industry amongst countries (Carter and Leonard 2002). We aimed to account for uneven sampling effort across regions and unsuccessful escape events by using a target-group approach for absence generating and by spatiotemporally analysing presence grid cells. Nevertheless, several escape events at a given site might lead to overestimation of persistence, whereas other regions due to scarcity of spatially explicit records might be under-represented and their suitability hence underestimated (e.g. Southern Europe). Due to these data limitations and because the coypu most likely has not yet colonised all climatically suitable regions in Europe, here, the calculated environmental niche might be conservative. Misclassification increased with increasing hilliness in a region, likely due the chosen spatial resolution, which is too coarse to properly characterise the full variation of environmental conditions in heterogeneous areas (e.g. valleys, which can locally provide suitable habitat in mountainous regions). In addition, a temporal change in sampling effort could lead to earlier decades being under-represented, hence, the impression of the species' spread could be intensified by more data becoming available recently, for example, through citizen science projects.

Implications for management

The majority of grid cells deemed suitable for coypu under current climate or climate change are not yet colonised. Our results illustrate the urgent need to not only improve management measures in areas with persisting populations, but also find strategies to prevent or reduce further spread as the costs of early intervention are much smaller than control of established populations (Panzacchi et al. 2007). Although the eradication of the coypu in Great Britain was successful, this was achieved as a result of coordinated intensive trapping efforts which were executed by employed professionals over one full decade (Gosling and Baker 1989). Moreover, Great Britain has the advantage of being an island and re-invasion is therefore unlikely. Considering the current situation on the European mainland, with widespread occurrence in Central Europe, it seems highly unrealistic to attempt total eradication of the species in Europe.

Baroch and Hafner (2002) argue that, in the case of low population densities, impacts of coypus in general are rather minor. Given that the coypu is already fairly widely distributed, management that minimises population density and therefore negative economic and environmental impacts should be the aim. As there are several hotspots of coypu occurrence covering more than one country, there is a need for international collaboration to coordinate control measures on a metapopulation scale and prevent compensatory re-invasion from adjoining populations (Oliver et al. 2016). There are well-known cases of migration events of coypus from neighbouring countries, for example, from France to Germany (DVWK 1997, Scheide 2013) and from Belgium and Germany to the Netherlands (Carter and Leonard 2002). Already existing binational control programmes could serve as best-practice examples. Gosling and Baker (1989)

suggest concentrating efforts on high-density hotspots to maximise mortality and minimise dispersal to new habitats. Further research regarding coypu dispersal movement and interaction on metapopulation level, also taking into account population genetics, would give new insights to its spreading history across Europe and allow the identification of relevant centres of dispersal.

Accounting for coypu in hunting laws would allow integrating it as a wildlife resource and harvesting coypu for its meat and fur. Meat of wild coypus was shown to be low in fat and cholesterol, while rich in proteins (Tulley et al. 2000) and the use of the coypu as a food source was common in Eastern Europe during the last century (Carter and Leonard 2002). Increasing the market value of the species would introduce an incentive for trappers and hunters and was shown to result in population decreases (Carter and Leonard 2002, Scheide 2013); however, it may also result in the wish to manage populations for permanent resource extraction.

Aside from direct control measures, another aspect of coypu management is the facilitation of winter survival and rise of reproduction rates by providing additional food sources. Wildlife feeding in or nearby settlements can induce rapid increases of coypu populations. Urban feeding sites and easily accessible agricultural areas may suffer from high coypu abundances (Walther et al. 2011, Scheide 2013). Managing urban populations is aggravated by the fact that the general public is often against lethal control methods of charismatic species, such as furry mammals (Walther et al. 2011, Jarić et al. 2020). Feeding bans, in combination with educational measures, such as awareness campaigns in such areas, are thus essential and have multiple positive benefits.

Conclusions

It is well-established knowledge that the coypu causes substantial economic and environmental damage when occurring at high densities. Although cool-temperate climates were believed to keep coypus at low densities, in many parts of Europe numbers have increased strongly during the last decades (Gosling and Baker 1989, Carter and Leonard 2002, Scheide 2013). Therefore, in Europe, the species was already subject to several national control campaigns (Carter and Leonard 2002), peaking in its inclusion as species of European Union concern (EU Commission 2014).

Our study shows that the coypu has, by far, not yet reached all potentially suitable regions in Europe and further highlights the importance of clarifying its response to increasing temperatures and arid conditions as they are likely to increasingly occur in the near future under climate change. However, one must consider the shortcomings of predictions that are made on the basis of opportunistic records from various sources and of differing data quality. Sampling effort differs spatiotemporally across the study area and, although we considered the violated assumption of an equilibrium for taxa undergoing an invasion process (Elith and Leathwick 2009, Václavík and Meentemeyer 2009, 2012) by depicting the uncertainty in predictions through a consensus approach, the outcome should be interpreted with caution (Pearce and Boyce 2006).

Predictions of invasion processes should be regularly reassessed, ensuring that eventual changes in the species realised niche are captured (Václavík and Meentemeyer 2012). Nevertheless, SDM-based predictions of alien species' distributions provide valuable tools to predict the next phase of invasion (Václavík and Meentemeyer 2012) and areas at high risk and can serve as the basis for further detailed analyses on regional or local scales, helping to better allocate resources for both surveys and management.

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Supplementary material I

Supplementary materials

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Data type: tables and figures

Explanation note: **Figure S1.** Pairwise Pearson's correlation coefficients for predictor variables. **Figure S2.** Absence sampling strategies. **Figure S3.** Predictive ability of the consensus prediction under current climatic conditions (1979–2013). **Figure S4.** Calculation of the weighting factor for the coypu invasion risk map. **Table S1.** Presence data sources included in this study, listed by country and the total number of records per country after data cleaning. **Table S2.** Top models ($\Delta AIC < 4$) that have been used for averaging within the persistence levels and their according weight.

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