Investigations into *Phytophthora* dieback of alder along the river Lagan in Belfast, Northern Ireland

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Abstract

Common alder (*Alnus glutinosa*) is an important tree species, especially in riparian and wet habitats, it is very common across Ireland and Northern Ireland, and provides a wide range of ecosystem services there. Alder suffers from *Phytophthora* induced decline in many parts of Europe, and this research set out to identify the presence and scale of the risk to alder health from *Phytophthora* and other closely related oomycetes in Northern Ireland. This was done through surveys along the river Lagan in Belfast, Northern Ireland and revealed that of the tree vegetation along an 8.5 km stretch of the river, 166 alder trees were counted. Of these, 28 were severely defoliated/diseased and nine were dead. Sampling and a combination of morphological and molecular testing of symptomatic plant material and river baits identified the presence of three *Phytophthora* species, including *Phytophthora lacustris* -the first time it has been recorded as disease-causing in Ireland. Inoculation studies using potted alder saplings demonstrated that *P. lacustris* was able to cause disease (under bark lesions), and Koch's postulates for this pathogen-host combination were completed, which suggests a future risk to alder health from *P. lacustris* in Northern Ireland.

Key words: *Plant health, forest pathology, riparian,* Alnus glutinosa *root and collar rot.*

Introduction

Common alder (*Alnus glutinosa* (L.) Gaertn.) is native to Europe, being common across Britain and Ireland (Clapham et al. 1952). Alder (*A. glutinosa* and *Alnus cordata*) accounts for 2.7% of the forest estate in Ireland (NFI 2017), and is known as a relatively short lived tree (ca. 150 years; Mitchell 1996) that is suitable for planting in sites prone to water logging (Horgan et al. 2004). Native alder woods are common on wet poorly drained sites in Ireland and Northern Ireland, and can support a diverse herb and bryophyte layer (Cross 2012, DAERA 2020). Planting of *Alnus* in forests is supported in Ireland and Northern Ireland by government forestry grants (DAFM 2015, DAERA 2019). Furthermore, it has been suggested that introducing

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Alnus into plantations of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) in Ireland and Britain would add structural diversity and have a positive effect on biodiversity and associated ecosystem services (Deal et al. 2014).

In 1993, a dieback of *Alnus* was noted in Britain, associated with a then unknown *Phytophthora* species (Gibbs et al. 1999). Further research identified the pathogen as being widespread in England and Wales (Streito 2003), and the pathogen was formally described as *Phytophthora alni* (Brasier et al. 2004). *Phytophthora alni sensu lato* was later split into three taxa, the species *Phytophthora uniformis*, and the hybrid species *Phytophthora* × *multiformis* and *Phytophthora* × *alni* based on molecular analysis (Husson et al. 2015). Dieback of alder caused by these pathogens has now been recorded in 17 European countries (Bjelke et al. 2016). Alder dieback was first confirmed in Ireland in 1999 (Clancy and Hamilton 1999), associated with *Phytophthora* × *multiformis* (O'Hanlon et al. 2016a), while findings of alder dieback associated with *P. uniformis* were made in 2016 (O'Hanlon et al. 2016b). All of these aforementioned pathogens infect trees via the root or root collar under natural circumstances (Jung et al. 2018). To date, none of the previously mentioned pathogens have been detected in Northern Ireland (O'Hanlon et al. 2016a), although dieback symptoms on alder have been evident for several years. For example, images on the free online resource Google street view from July 2008 to July 2018 show signs of alder dieback (e.g. thinning crown, bleeding cankers on the trunk, tree mortality) along the river Lagan in Belfast Northern Ireland UK (Figure 1).

In 2017, an alder tree with typical *Phytophthora* dieback symptoms (i.e. black tar-like bleeding trunk cankers, thinning canopy; Jung et al. 2018) was discovered along the river Lagan near the Agri-Food and Biosciences Institute, Newforge lane, Belfast BT95PX (Figure 2). In order to investigate this disease and its potential impact on other alder trees in the area, this research set out to:

(I) identify the cause of the disease on the symptomatic tree;

(II) conduct live plant inoculation studies to confirm the pathogenicity of *Phytophthora lacustris* to *Alnus glutinosa*;

(III) sampling the river using leaf baiting to identify if *Phytophthora* species were present in the water; and

(IV) carrying out an alder health survey along a portion of the river. *Phytophthora* are known to be water borne pathogens (Zappia et al. 2014), therefore the water baiting method and the survey along the river were used to record the distribution and spread of the pathogen.

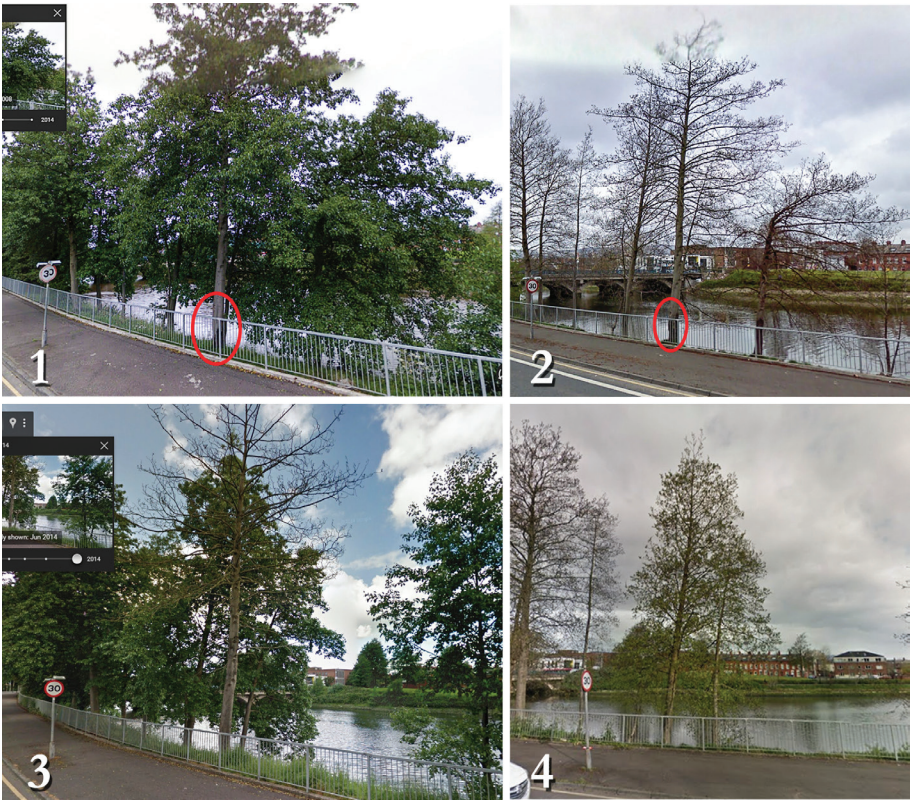

Figure 1*: Chronosequence of Google street-view images (composites) showing symptoms of alder dieback along the river Lagan in Belfast, Northern Ireland (54°34'58.1" N, 5°55'12.8" W). The images were taken in different months (starting from image 1 to 4, respectively): July 2008, April 2010, June 2014, and April 2017. Areas showing typical Phytophthora bleeding cankers have been encircled in a red oval. A thinning canopy is another common symptom associated with Phytophthora dieback of alder and this can be seen in images 1 and 3. The focal infected tree is not present in image 4, presumably having been removed by either flooding or remedial works.*

Methods

Riparian tree visual survey

In order to investigate the extent of potential alder decline along the river, a visual survey of tree conditions was carried out along an 8.5 km stretch of the river. Mortality (dead or living), the level of crown defoliation (classed using a numerical scale of 1 for low defoliation to 4 for extensive defoliation; Lakatos et al. 2014), and size class (1 = Diameter at Breast Height (DBH) <10 cm, 2 = DBH 10 – 20, 3= DBH >20) and the presence of cankers for every alder tree was recorded. Notes were also taken of other tree health aspects for the surveyed alder trees. To get a rough estimation of the other tree species present in the area, the neighbouring species of tree to the left and

Figure 2: *Bleeding cankers (encircled in red on the image) on an alder tree from which* Phytophthora lacustris *isolate P17-120 was collected. The cankers were all less than 30 cm diameter, and appeared either as wet oozing lesions or as dried crusted mounds.*

right of each alder tree were also recorded, if they were not also alders. The health condition of the neighbouring trees was not studied.

Symptomatic tree sampling and testing

Although several alder trees with *Phytophthora*-like symptoms were noted in the survey, just one symptomatic tree (Figure 2; 54°33'26.9" N, 5°56'10.7" W) was sampled due to an inability to safely sample the other trees as they were in precarious river bank positions. For the sampled tree, bark samples 1 cm think (including the cambium) from the upper and lower limits of bleeding cankers were taken using a mallet and wood chisel. Samples were surface sterilized and processed according to O'Hanlon et al. (2016b). Briefly, samples were put onto PARP agar (1 litre distilled water, 17 g cornmeal agar, 10 mg Pimiricin, 250 mg Ampicillin, 10 mg Rifamycin, 100 mg PCNB; after Jeffers and Martin (1986)) and incubated at room temperature (19 °C) for up to two weeks and monitored for *Phytophthora*-like growth. Any *Phytophthora*–like mycelium was aseptically transferred onto carrot piece agar (CPA; Werres et al. 2001) and incubated for five days at 19 °C. The isolate (P17-120) was examined morphologically, noting the colony morphology and spore characteristics of general guides (e.g. Gallegly and Hong 2008). To induce sporangia production to aid in morphological identification, pieces of the outer colony edge were cut from the CPA and put into a clean petri dish and flooded with non-sterile pond water (i.e. a mixture of 50% water from the river Lagan and 50% tap water) to induce spore formation according to Scanu et al. (2015). The pond water was >6 months old at the time, in order to provide some assurance that any *Phytophthora* spores that may have been in the water were dead. Zappia et al. (2014) reviewed oomycete risks from water and indicate that in general, survival for more than a few days is uncommon. DNA sequencing of the ITS gene region using the primers ITS4 and ITS5 primers (White et al. 1990) was carried out on a pure culture isolate on CPA. The DNA sequence was checked against the database of curated *Phytophthora* sequences in PhytophthoraID (Grünwald et al. 2011), and also against the uncurated reference database available in GenBank using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). The isolate from the symptomatic alder tree (P17-120) was deposited in GenBank under the accession number MH784622. This isolate was stored in culture at 4 °C for several weeks until use in pathogenicity testing. Prior to using it in pathogenicity tests it was grown on PARP for 1 week followed by transferring to CPA and growing for a further 1 week.

Water baiting methods

At two points along the Lagan, leaf bait bags were deployed according to Turner et al. (2006) and O'Hanlon et al. (2018) with minor modification as follows: a leaf of

A. glutinosa, *Rhododendron ponticum*, and *Quercus petraea* (Matt.) Liebl. was removed from live, symptomless (i.e. putatively disease free) plants on the day of baiting, and sealed into a bait bag. Three hosts were used to increase the chances of collecting a diverse oomycete assemblage (Jung et al. 2020). No comparisons were carried out to assess the efficacy of the different bait species for collecting oomycetes. The bait bag was composed of a double layer of muslin cloth, which was stapled shut, tied to the river bank with fishing line, and dropped into the river and left to float in the current. Bait bags were collected after 1 week, returned to the lab and the leaves removed from the bag. The process for isolating and identification of any *Phytophthora*-like cultures from the bait leaves was the same as for the symptomatic tree sampling and testing above.

Pathogenicity testing

In order to prove that a pest is able to cause disease in a host, the four criteria of Koch's postulates (as translated by Rivers 1937) must be satisfied. These criteria prove that the pest alone is the cause of the disease, and help greatly in highlighting the risk to that host from the pest. Potted seedlings of *A. glutinosa* (<3 years old) that were visually disease free were purchased from Coillte nurseries in Co Carlow, of which 12 were inoculated with agar plugs taken from the edge of the *P. lacustris* colony (P17-120) growing on CPA. Five other *A. glutinosa* seedlings were inoculated with sterile CPA plugs to act as a negative control. The experiment was carried out as follows: in July 2018, each plant was wounded with a 3-cm cut into the bark approximately halfway up the stem, with the agar plug inserted into the cut and sealed with damp cotton wool and parafilm. The plants were kept outside in a dedicated experimental area and watered regularly. After 6 weeks the outer bark was carefully scraped off and the lesions examined and measured with a tape measure. Data describing lesion lengths of replicates for each treatment (inoculation vs control) were plotted and compared visually for differences between treatments according to the method of Ho et al. (2019) using the online tool available at https://www.estimationstats.com. This statistical tool plots the frequency distribution of the mean difference between the treatments. This uses 5,000 bootstrap samples to provide confidence intervals for the data and analysis. The entire diseased area in each plant was removed with a sterile knife, and transferred to the laboratory for *Phytophthora* isolation as per the above methodology.

Results

Alder survey

A total of 166 alder trees were surveyed along an 8.5 km stretch on one side of the Lagan. The majority (83% or 137 trees) had diameters greater than 10 cm (Table 1). Of the total number of alder, 121 had low defoliation (1 or 2), with 28 showing moderate

Table 1: *Results of the riparian alder survey. Tree species other than alder were only recorded if they were growing next to an alder tree. The presence of cankers was only assessed on alder.*

Tree species	No. of trees recorded	Number with cankers
Sycamore (*Acer pseudoplatanus* L.)	9	N/A
Willow (*Salix* spp.)	14	N/A
Birch (*Betula* spp.)	3	N/A
Ash (*Fraxinus excelsior* L.)	23	N/A
Beech (*Fagus sylvatica* L.)	3	N/A
Oak (*Quercus* spp.)	5	N/A
Poplar (*Populus* spp.)	3	N/A
Hawthorn (*Crataegus monogyna* Jacq.)	8	N/A
Hazel (*Corylus avellana* L.)	1	N/A
Lime (*Tilia cordata* Mill.)	1	N/A
Alder size class 1[a]	29	2
Alder size class 2[b]	121	5
Alder size class 3[c]	16	2

[a] Diameter at Breast Height (DBH) <10 cm
b DBH 10-20cm
c DBH >20cm

to severe defoliation. Most of these were in the middle size class. Nine *Alnus* trees were dead. Nine of the trees had bleeding cankers, and all of these were in defoliation class 3 or 4. The bleeding cankers were all small in diameter (>1 cm), were dried in appearance, and sparsely scattered on trees usually numbering <4 per affected tree. The bleeding cankers were only found on the main trunk of trees.

Phytophthora *isolation and characterisation*

The isolate (P17-120) from the symptomatic *Alnus* tree was fast growing and produced a petaloid colony morphology similar to *Phytophthora gonapodyides*. The isolate produced non-caducous, non-papillate, mainly ovoid (some obpyriform) sporangia (average 35 × 25 µm). Sporangia showed internal and external proliferation and often had wide exit pores. Comparison of the DNA sequence from this isolate with PhytophthoraID indicated that the isolate was a very close match (99%) to *Phytophthora lacustris* (Table 2).

Leaf baiting

Over 10 weeks of baiting, with weekly collections from 2 locations, 159 isolates were collected. These isolates were grouped into 4 morphogroups based on gross colony morphology, and spore microscopic characteristics. Morphotypes 1 – 3 were typical *Phytophthora*-like colonies, with varying rosette/petaloid gross colony morphology

Table 2: *Results of the DNA sequencing of representative isolates of each of the morphogroups*.

Sample	Best match PhytophthoraID (accession number; % similarity)	Best match GenBank (accession number; % similarity)	No. isolates collected
Symptomatic *Alnus* canker (P17-120)	*Phytophthora lacustris* (AF266793.2; 99)	*P. lacustris* (MT328701.1; 100)	1
Baiting morphotype 1	*P. lacustris* (AF266793.2; 96)	*Phytophthora gonapodyides* (MF034095.1; 96) / *P. lacustris* (MG721454.1; 96)	3
Baiting morphotype 2	*Phytophthora chlamydospora* (AF541901.1; 99)	*P. chlamydospora* (MN513235.1; 99)	13
Baiting morphotype 3	*P. lacustris* (AF266793.2; 97) / *P. chlamydospora* (HM004224.1; 97)	*P. lacustris* (MG721454.1; 97) / *P. gonapodyides* (MF034095.1; 97)	91
Baiting morphotype 4	*Pythium vexans* (AF271224.1; 80)	*Phytopythium litorale* (MK015677.1; 99)	52

and varying levels of fluffy appearance due to aerial mycelium growing up from the agar surface. Morphotype 4 was relatively fast growing with thin hyphae and in some cases ornamented circular oospores. DNA sequencing indicated that these morphogroups represented four different taxa, although the % similarity between the isolates collected and those in the online databases varied (Table 2). Three of these morphotypes (Morphotype 1, 2, 3) showed equal similarity in DNA sequence to more two different species in the Genbank database. For these isolates it is not possible to definitively identify the species. Morphotype 3 was commonly isolated, with morphotype 1 only isolated on 3 occasions.

Pathogenicity testing

After 6 weeks of the experiment dark lesions around the inoculation point were observed. The plants inoculated with *P. lacustris* isolate P17-120 had lesions of mean length 4.4 cm (n = 12, SD+/- 0.76) whereas control plants had lesions of mean length 3.42 (n = 5, SD+/- 0.60) (Figure 3). This significant difference between mean lesion size of the two treatments was confirmed by a Mann Whitney U test ($U = 51.5$, $p < 0.05$). Attempts to isolate *Phytophthora* from the wounded points on inoculated and control saplings were carried out, with *P. lacustris* (confirmed by morphology) re-isolated from the necrotic tissue from all of the inoculated trees. No *Phytophthora* cultures were isolated from control plants. This completes Koch's postulates for this host-pathogen pair in Northern Ireland.

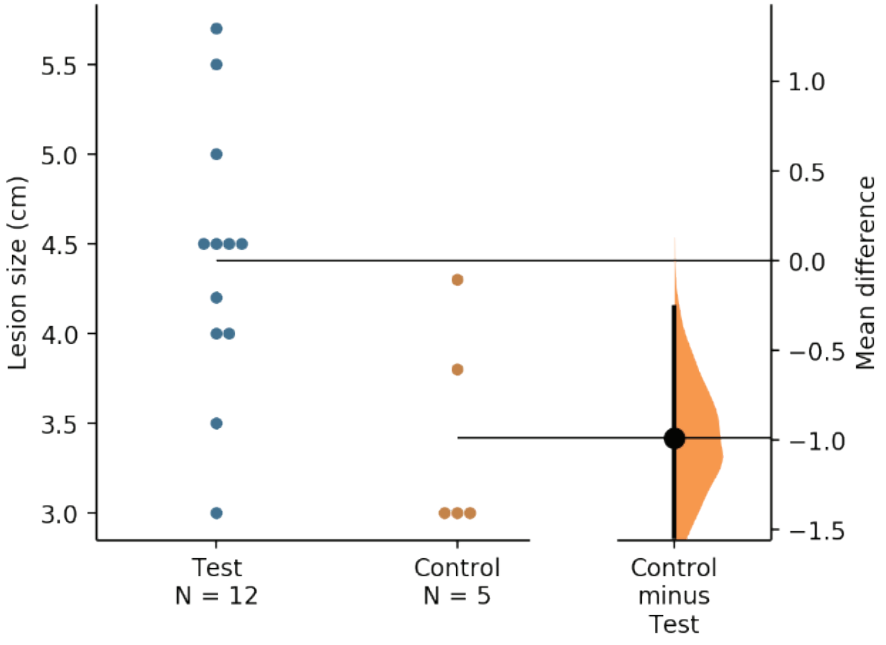

Figure 3: *Estimation plots of data form the pathogenicity test. Saplings were inoculated with* Phytophthora lacustris *(test) or with sterile agar plugs (control). The filled curve indicates the complete difference in means distribution, given the observed data. The 95% confidence interval of difference in means is illustrated by the thick black line. The difference in means between the treatments (inoculation with* P. lacustris *or control) was significantly different based on a Mann Whitney U test (U = 51.5, p <0.05).*

Discussion

Alder dieback due to *Phytophthora* is currently widespread along river systems in many European regions including southern Germany (Jung and Blaschke 2004), Northern France (Thoirain et al. 2007), Czech Republic (Černý and Strnadová 2010), Sweden (Redondo et al. 2015), and England (Webber et al. 2004). The disease is also present in Ireland (O'Hanlon et al. 2016a), though large scale surveys are needed to delimit its extent. The resulting alder mortality in these river systems will likely have many negative impacts on the ecosystems services these trees provide (Černý et al. 2008, Bjelke et al. 2016). Alder has many important functions in riparian habitats, including provision of shade, nitrogen fixation, stabilization of river banks through root networks, provision of habitat for many organisms, and increases in the aesthetic appeal of river banks (Bjelke et al. 2016). In Ireland and Northern Ireland, it is the 6th most commonly recorded tree species in the wild, after *Crataegus* spp., *Fraxinus excelsior*, *Acer pseudoplatanus* L., *Ilex aquifolium* L., and *Prunus spinosa* L. (National Biodiversity Data Centre 2019).

Jung et al. (2018) provide a comprehensive review of the distribution of alder decline in Europe. They highlight that although the main cause of the decline is

usually either *Phytophthora uniformis*, or one of the hybrid taxa *Phytophthora* × *alni* or *Phytophthora* × *multiformis*; other *Phytophthora* species are also known to cause disease in alder. These include *Phytophthora cactorum*, *P. gonapodyides*, *Phytophthora plurivora*, *Phytophthora polonica* and *P. lacustris*. The isolate collected from the symptomatic alder tree in this study matched the description of *P. lacustris* (Nechwatal et al. 2013), while the majority of the isolates from the stream baiting were putatively identified as *P. lacustris*. Pathogenicity tests showed that *P. lacustris* caused disease in infected alder plants. *Phytophthora lacustris* has also been found infecting *A. glutinosa* in Portugal (Kanoun-Boule et al. 2016), as well as several other plant hosts in the European and Mediterranean region (Akilli et al. 2013; Nechwatal et al. 2013). The river baiting carried out in the study also indicates that there is a risk of *P. lacustris* spreading along the river and infecting more trees. This is similar to findings of *P. lacustris* in soil and streams in Ireland (O'Hanlon et al. 2016b). It is well known that *Phytophthora* species spread readily in water in both commercial (Zappia et al. 2014) and wider environment (Sims et al. 2015) locations. Gibbs et al. (1999) also found evidence that *Phytophthora* disease of alder in England was being spread in the river. Worryingly, the low number of young alder trees recorded in the survey may indicate that *P. lacustris* is reducing recruitment of young alder trees, as well as killing older ones. Younger plants are known to be more susceptible to *Phytophthora* infection than older ones (Oßwald et al. 2014). This could lead to a situation where *Alnus* becomes less common along the river Lagan, thus reducing the ecosystem services provided by *Alnus*. Whether the remaining tree species in the area can replace the alder in the future is not clear, as many of the other tree species are known to be susceptible to *Phytophthora* pathogens. *Phytophthora*-like symptoms have not been noted on other trees in the area (R. O'Hanlon unpublished data), though more thorough examinations of other trees in the area are warranted.

There were a number of limitations in the methods used in this study which need to be considered when interpreting the findings. Firstly, this study used a combination of morphotyping and DNA sequencing to identify the oomycete diversity. Unfortunately, the ITS DNA primers used did not allow for identification of the *Phytophthora* species to a conclusive level (i.e. >99% similarity). O'Hanlon et al. (2016b) also discussed this issue, when their sequencing was unable to separate closely related taxa within *Phytophthora* clade 7. Multilocus analysis based on the sequencing of more than one gene region is recommended to separate closely related species within the genus *Phytophthora* (Hyde et al. 2014, Yang and Hong 2018). The result of the DNA analyses also depended on the database used. Since PhytophthoraID is a curated database which only includes sequences of deposited species confirmed by competent taxonomists (Grünwald et al. 2011), this resource is likely to be more reliable and accurate. GenBank on the other hand

is not curated and is known to suffer from erroneous names of sequence deposits of other microorganisms (e.g. fungi; Nilsson et al. 2006) in the database. Although it is highly likely that the four morphotypes identified from the baiting samples were *P. gonapodyides*, *P. chlamydospora*, *P. lacustris* and *Phytopythium litorale*, this is not certain. However, these species have been found in surveys in Ireland (O'Hanlon et al. 2016b), and also in other samples from watercourses in Northern Ireland (O'Hanlon 2017). They are also common in other regions of the world (Černý et al. 2015, Duarte et al. 2015, Hansen et al. 2015). None of these species, except *P. lacustris*, is considered a serious threat to plant health in Europe (Jung et al. 2016), and they probably function as saprotrophs in speeding up the decay of plant material in streams (Hansen et al. 2012). Another limitation was that no attempt was made to isolate *Phytophthora* from other symptomatic trees. Attempts to isolate from other alder trees could lead to findings of active *Phytophthora* infection in other trees and further explain the threat to alder from *Phytophthora* species in this site.

It is interesting to speculate if, and how, *P. lacustris* may have entered the area. It is not known if *P. lacustris* is native to Ireland and Northern Ireland, since Jung et al. (2016) were unable to assign its putative native range. The species has been linked with plant trade infrequently across Europe (Jung et al. 2016) and the USA (Bienapfl and Balci 2014), and therefore could have been introduced inadvertently into Northern Ireland on infected plant material or soil. Trees along the Lagan River have almost certainly been planted as part of the normal maintenance works of the local councils. Alder trees are grown in tree nurseries in both Northern Ireland and Ireland, but the source of any trees that may have been planted in this area is unknown. The area of the Lagan surveyed in this research is highly modified by humans, with the nearby Belvoir forest area having a long history of exotic plant introductions. The riverbank also has a widespread infestation of *Rhododendron ponticum* L., Japanese knotweed (*Fallopia japonica* Houtt.), giant hogweed (*Heracleum mantegazzianum* Sommier and Levier), and Himalayan balsam (*Impatiens glandulifera* Royle). The nearby Belvoir forest also has had recent infestations of other important *Phytophthora* species, including the EU-regulated *Phytophthora ramorum* on Japanese larch (*Larix kaempferi* (Lamb.) Carr.) and the previously nationally regulated *Phytophthora lateralis* on Lawson Cypress (*Chamaecyparis lawsoniana* (A.Murray bis) Parl.). Coupled with this, there has been an invasion and outbreak of the non-native insect pests horse chestnut leaf miner (*Cameraria ohridella*) on horse chestnut (*Aesculus hippocastanum* L.) (Anon 2014) and ash sawfly *Tomostethus nigritus* on ash (*F. excelsior*) in the area since 2016 (Jess et al. 2017). Spread of *Phytophthora* by other human enterprises (e.g. fish farming; Jung and Blaschke 2004) and even by birds (Dadam et al. 2020) has been suggested elsewhere in Europe, though the evidence for significant spread by either of these means has not been shown.

Conclusions

This investigation is the first to demonstrate *P. lacustris* as a cause of disease in *Alnus* on the island of Ireland (i.e. in Northern Ireland or Ireland). Though we did not detect either of the other serious *Phytophthora* pathogens of alder (*P. uniformis* or *P.* × *alni*), it is also likely that they are responsible for alder decline in Northern Ireland, as these have already been recorded infecting *Alnus* in Ireland (O'Hanlon et al. 2016a). These results provide some evidence for a threat to alder in commercial forestry in Northern Ireland and Ireland, though studies in forest settings are needed to assess the relative importance of this risk. The ecology of the river ecosystem of the river Lagan is under threat from *Phytophthora* and many other invasive species. Further baiting, surveys and the use of novel surveillance methods (e.g. Google street view) will help understand the threat to riparian and urban tree health from *Phytophthora* and other plant pests.

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