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Detection and spread of *Phytophthora austrocedri* within infected *Juniperus communis* woodland and diversity of co-associated Phytophthoras as revealed by metabarcoding

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Abstract

Phytophthora austrocedri is a recently invasive soilborne pathogen which is causing widespread mortality of *Juniperus communis* in northern Britain. The pathways by which a single genotype of *P. austrocedri* has spread to infect such a geographically dispersed range of woodland sites within a relatively short timeframe are unknown. This study examined the detectability of *P. austrocedri* in soil and water within infected *J. communis* woodland using qPCR to gain a better understanding of the pathogen's key mechanisms of spread. A *Phytophthora* metabarcoding method was also applied to investigate the wider diversity of *Phytophthora* species present in water at one of the sites. qPCR analyses of *P. austrocedri* in soil samples at a *J. communis* woodland exhibiting low-to-moderate levels of disease suggested a slow natural spread of the pathogen in soil, requiring high moisture conditions. However, the ubiquity of *P. austrocedri* DNA in soil samples collected across a heavily infected *J. communis* site suggests that once established at a site the pathogen can be spread readily in soil locally, most likely vectored by animal movements and/or human activities. The hypothesis that *P. austrocedri* is aerially transmitted in rainwater was not adequately proven, and an alternative hypothesis for the widespread distribution of the pathogen on *J. communis* in northern Britain is presented. Metabarcoding identified DNA from a diverse range of *Phytophthora* species in river and rainwater samples although the main target pathogen, *P. austrocedri*, was not amplified which disagreed with some of the qPCR findings. Possible reasons for this are discussed.

KEYWORDS
juniper, molecular detection, *Phytophthora* spread

1 | INTRODUCTION

Phytophthora is a diverse genus of filamentous oomycete plant pathogens well known for causing root and foliar diseases on a very broad range of woody and herbaceous hosts. Currently, about 180 species have been described worldwide, phylogenetically split across ten (Yang, Tyler, & Hong, 2017) or twelve (Jung et al., 2017) clades. *Phytophthora austrocedri* (synonym: *Phytophthora*

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austrocedrae Gresl. & E.M. Hansen, sp. nov.) is a homothallic, clade 8 species first described in 2007 from southern Argentina where a clonal lineage known as the ARG lineage (Henricot, Pérez-Sierra, Armstrong, Sharp, & Green, 2017) is associated with widespread mortality of the native cypress *Austrocedrus chilensis* (Cupressaceae) (Greslebin & Hansen, 2010; Greslebin, Hansen, & Sutton, 2007). In 2011, a second genetically and morphologically distinct clonal lineage (UK lineage) (Henricot et al., 2017) of *P. austrocedri* was confirmed causing dieback and mortality of *Juniperus communis* (common juniper) in northern England (Green, Hendry, MacAskill, Laue, & Steele, 2012). *Juniperus communis* is a dioecious evergreen conifer with a broad boreo-temperate distribution stretching to 30 °N throughout northern Asia, North America and Europe (Preston, Pearman, & Dines, 2002; Thomas, El-Barghathi, & Polwart, 2007). In Britain, *J. communis* is one of only three native conifer species and is distributed in generally fragmented populations across the country (Preston et al., 2002). In Scotland in particular, *J. communis* is highly valued as an important constituent of *Betula*, *Quercus* and *Pinus* woodland ecosystems. For this reason, *J. communis* is listed as a priority species in the UK Biodiversity Action Plan [http://jncc.defra.gov.uk/ukbap and http://archive.jncc.gov.uk/page-5171]. Following the first discovery of *P. austrocedri* in northern England, a detailed survey of *J. communis* populations across northern Britain revealed that *P. austrocedri* had a surprisingly widespread distribution, causing root and stem infections at well over one hundred geographically separate sites and contributing to a severe decline of this ecologically important native conifer species (Green, Elliot, Armstrong, & Hendry, 2015). At several sites, the presence of long-dead, skeletal trees within infected areas suggested that the pathogen had been present for around a decade or possibly longer.

There is no firm evidence documenting how *P. austrocedri* first entered Argentina or Britain and the geographical origin of *P. austrocedri* is unknown. In Argentina, mortality of *A. chilensis* was first detected in 1948 on Victoria Island which was known for its introduction of exotic woody plants from different continents. This has led to the view that *P. austrocedri* was introduced into that country on infected, imported plants (Vélez, Coetzee, Wingfield, Rajchenberg, & Greslebin, 2013). DNA of *P. austrocedri* has been found in diseased tissues of young *Juniperus* species, *C. lawsoniana* and *Cupressus* x *leylandii* imported into Britain from other European Union countries (J. Barbrook, Animal and Plant Health Agency, York, England, personal communication and A. Schlenzig, Science and Advice for Scottish Agriculture, Edinburgh, Scotland, personal communication) and in diseased *J. communis* plants located in nurseries and private gardens in England and Wales (Denton, Denton, Waghorn, & Henricot, 2010). Therefore, the international plant trade is likely to be a key pathway for the transcontinental spread of this pathogen with all known hosts to date residing in the Cupressaceae.

In terms of intra-country spread, it is currently not known how a single genotype of *P. austrocedri* has come to infect so many geographically distinct sites in Britain within a time frame indicated by disease observations to be from the 1990s onwards. The thick-walled, resilient oospores which this pathogen forms readily in vitro are a primary mechanism by which many *Phytophthora* species exist for long periods in soil without a living host (Ristaino & Gumpertz, 2000). DNA of *P. austrocedri* has been detected in soil collected around infected *J. communis* (Elliot, Schlenzig, Harris, Meagher, & Green, 2015; Riddell et al., 2019) as well as a number of public garden and amenity woodland sites in Scotland (Riddell et al., 2019). Longer-distance dispersal could be facilitated by the inadvertent transfer of infested soil and root fragments through human or animal activity, as has been shown for several *Phytophthora* species (Davidson, Wickland, Patterson, Falk, & Rizzo, 2005; Elliot et al., 2015; Hansen, Goheen, Jules, & Ullian, 2000). The potential of *P. austrocedri* to contaminate soil across infected sites needs to be ascertained before the risk of spread of the pathogen by transfer of soil can be understood.

Conversely, one argument against *P. austrocedri* having spread within the UK via soil infested with oospores, which are sexually produced, is the clonal nature of the isolates collected to date. Variation in heterozygosity would be expected, even in a homothallic species. The lack of such variation suggests that the method of dispersal of *P. austrocedri* in Britain has been purely asexual (Henricot et al., 2017). This could occur via movement of the waterborne zoospores in rain or river water or hyphae in infested plant material. Since *P. austrocedri* produces non-caducous sporangia which are not readily detached, it is not considered to be an aerially dispersing species able to be spread by wind-driven rain. However, aerial lesions with no connection to the base of the tree are not uncommon on *J. communis* (Green et al., 2015) and so it is possible that rain splash or possibly even bird-vectored transfer might allow a certain level of aerial dissemination.

Previous investigations have found other *Phytophthora* species associated with *P. austrocedri*-infected *J. communis* woodlands in northern Britain, including *P. cambivora*, which was detected by PCR and sequencing in basal lesions of two *J. communis* for which a qPCR test for *P. austrocedri* was negative (S. Seddaiu, Forest Research, UK, unpublished data). Riddell et al. (2019) applied an Illumina metabarcoding approach to analyse *Phytophthora* diversity in soils collected from fourteen public garden and woodland sites in Scotland. The authors detected six *Phytophthora* species from around ten symptomatic and asymptomatic *J. communis* at one woodland site, including *P. austrocedri*, *P. cactorum*, *P. cambivora*, *P. gonapodyides*. *P. pseudosyringae and P. ramorum*. The study demonstrated the power of metabarcoding for analyses of *Phytophthora* diversity in soil samples (Riddell et al., 2019), but the approach has not been used to analyse the diversity of species in rain and river water which might give a different picture of species abundance, ability to spread and potential for species' interactions.

The main aim of this study was to examine the detectability of *P. austrocedri* in soil and water at infected *J. communis* woodlands in order to gain a better understanding of how this pathogen spreads. A second aim was to apply the *Phytophthora* metabarcoding method previously demonstrated on soil samples (Riddell et al., 2019) to investigate the wider diversity of *Phytophthora* species present in

water samples. The study was designed to test the following hypotheses: (a) at a heavily infected site, higher levels of *P. austrocedri* DNA in the soil can be related to proximity to symptomatic *J. communis* and lower elevation; (b) at a less-infected site, *P. austrocedri* will be detected in fewer soil samples as distance increases from an infection point; (c) *P. austrocedri* is transmitted aerially, thus explaining its apparent asexual spread into a geographically diverse range of wild *J. communis* populations within a relatively short time period; and (d) the diversity of *Phytophthora* species detected in water samples at a heavily infected site will reflect the diversity of species previously found in soil samples at the same site by Riddell et al. (2019). To do this, we employed a quantitative real-time PCR assay specific to *P. austrocedri* (Mulholland, Schlenzig, MacAskill, & Green, 2013) to analyse both soil and water samples for the presence of the pathogen and additionally tested the metabarcoding approach of Riddell et al. (2019) on water samples. The results are discussed in the context of understanding how *P. austrocedri* has become so ubiquitous across northern Britain and the potential role of other *Phytophthora* pathogens detected by metabarcoding.

2 | MATERIALS AND METHODS

2.1 | Extent of *P. austrocedri* infestation of soil at site 1

Site 1, which was chosen to investigate the extent of *P. austrocedri* infestation of soil, is a heavily infected 100 Ha *J. communis* woodland and designated Site of Special Scientific Interest (SSSI) located on a north-facing slope in Perthshire, Scotland (Figures 1, 2). In June 2015, soil samples were collected from along each of three transects running north to south uphill on the site, with transects positioned in areas of high density of *J. communis* trees exhibiting foliage condition ranging from fully healthy, to partially bronzed, to dead. The first sampling point on each transect was located approximately 50 m below the start of the *J. communis* woodland at the lower elevation (northern) boundary of the site, and the last sampling point was located approximately 2–5 m above the *J. communis* woodland at the higher elevation (southern) boundary of the site (Figure 2). Transect 1, which was easternmost, ran from 180 m to 300 m elevation; transect 2, which lay to the west of transect 1, ran from 185 to 310 m elevation; and transect 3, which lay towards the western end of the site, ran from 210 to 330 m elevation (Figure 2).

Soil samples (approximately 300 g) were collected from 10 points per transect at approximately 10-m intervals and were comprised of four pooled soil cores of 2 cm width x 30 cm depth collected using a soil auger within a 1-m^2 area at each point on the transect. For each sampling point, the geographical coordinates were recorded as well as the approximate distance from the nearest *J. communis* and its state of health, which was documented using three categories: 1 = healthy, 2 = partial foliage dieback/bronzing and 3 = dead. The four soil subsamples were pooled after discarding any vegetation, homogenized by hand in a single grip-seal™ polythene bag and stored at 4°C overnight. Each soil sample was then oven-dried at ~60°C in aluminium trays for 1–3 d (depending on soil wetness), stirred thoroughly once dry and DNA extracted from three 250 mg subsamples using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc.). A robotic workstation for DNA extraction based on magnetic particle purification (Kingfisher™ mL Magnetic Particle Processor, Thermo Scientific) was used for the DNA extraction process. Post-DNA extraction clean-up was carried out using either the Jet-Quick™ DNA Purification Kit (Genomed GmbH) or DNA Clean & Concentrator™ (Zymo Research) according to the manufacturer's instructions.

Real-time PCR amplification was performed in TaqMan Environmental Mastermix 2.0 (Applied Biosystems) in 20 µl reaction volumes containing 2 µl template DNA. PCR was carried out in an ABI Prism 7,300 Real-Time PCR System (Applied Biosystems). Real-time PCR was conducted using *P. austrocedri*-specific

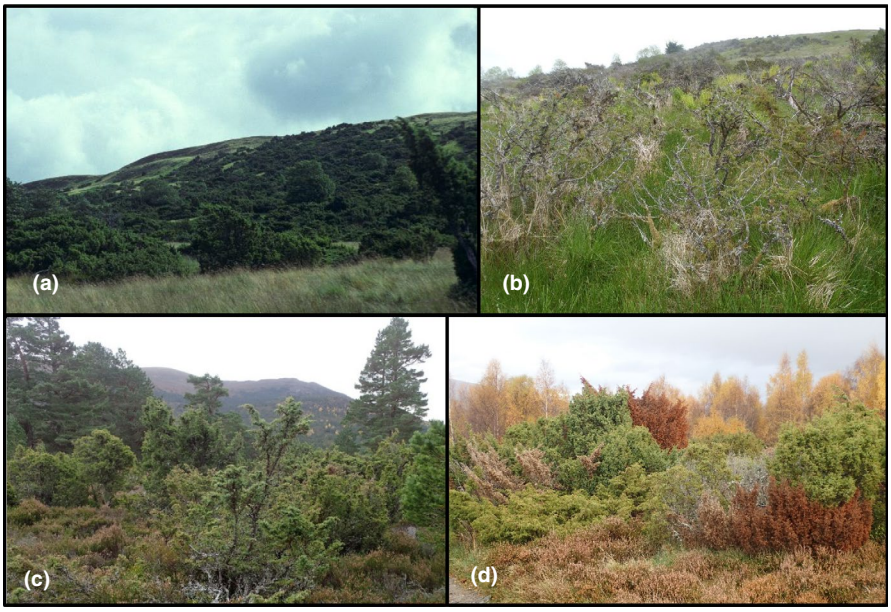

FIGURE 1 *Juniperus communis* at (a) site 1 in 1979 before the spread of *P. austrocedri* across the site, (b) site 1 taken from a similar perspective as in a) in 2015 showing extensive mortality caused by *P. austrocedri*, (c) site 2 within the Caledonian pine forest and (d) site 2 showing range of symptoms caused by *P. austrocedri*

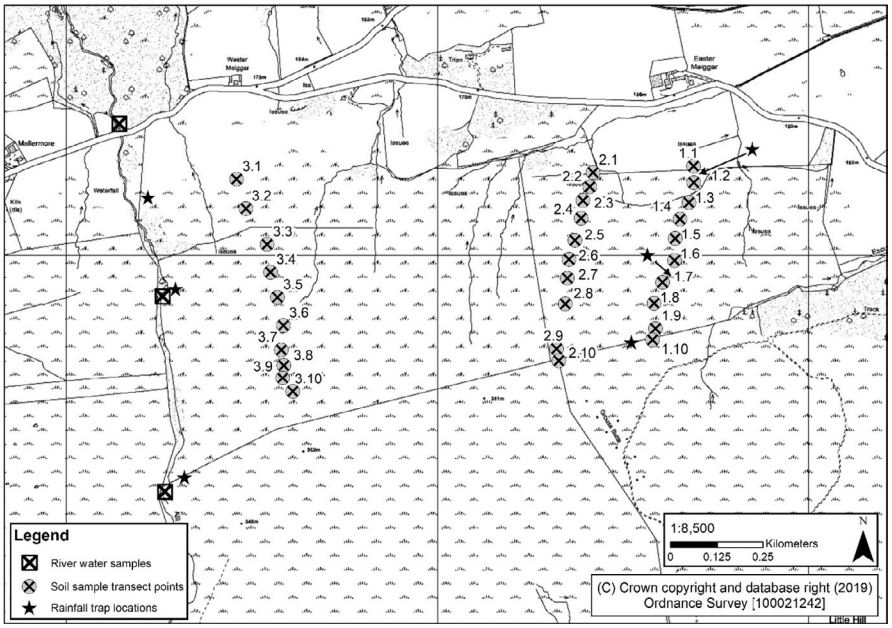

FIGURE 2 Map of site 1 showing location of sampling points. The area occupied by *J. communis* is bounded by the sampled stream to the west, the straight fence line indicated as running from the south-west in a north-easterly direction and the road which runs across the north of the map

primers Paus-481-F TGGTGAACCGTAGCTGTATTTAAGC, Paus-554-R GGAACAACCGCCACTCTACTTC and probe Paus-507-TM TGGCATTTGAACCGRCGATGTG following the protocol described by Mulholland et al. (2013). For each real-time PCR run, a standard curve was generated for a set of samples containing 200, 20, 2 and 0.2 pg DNA extracted from pure colonies of a single isolate of *P. austrocedri* whereby 0.2 pg DNA gave a Ct value of around 33. Each soil sample was tested in triplicate within a single real-time PCR run. For all real-time PCR runs, three negative control reactions containing molecular grade water instead of DNA template were included to check for contamination. Statistical analysis of the amount of target DNA amplified in each qPCR was conducted in R version 3.5.1 (R Core Team, 2018), using ggplot2 for data visualization (Wickham, 2016). Data were not normally distributed (positively skewed) and therefore were natural log-transformed prior to regression analysis, with main effects of transect, sample position and the interaction of juniper condition and distance to nearest juniper. A stepwise removal, based on AIC values, was used to refine the model predictors, with statistical significance determined using analysis of variance (ANOVA) (Fox & Weisberg, 2011). Post hoc analysis (Tukey's HSD) (Lenth, 2018) was used to determine marginal means for significant effects.

2.2 | Extent of radial spread of *P. austrocedri* from infection foci at site 2

Site 2, which was chosen to investigate the distance of radial spread of *P. austrocedri* in soil from an infection point, is a 130 Ha *J. communis* woodland located in the Cairngorm area of Inverness-shire, Scotland (Figure 1). At the time of sampling, this site exhibited low-to-moderate levels of infection caused by *P. austrocedri* which was first isolated from the site in 2015. Three *J. communis* trees were selected as the central points for soil sampling based on exhibiting different stages of disease. All three trees were located in a part of the site where *J. communis* is the dominant species. These were as follows: tree 1, which was skeletal dead and located in waterlogged soil; tree 2, which was dead with bronzed, retained foliage; and tree 3, which was live but exhibited partial crown dieback and a cinnamon-brown lesion in the phloem at the lower stem typical of *P. austrocedri*. All trees were located at around 320 m elevation and, in the case of trees 1 and 2, at least 5 m from the nearest symptomatic *J. communis*. Tree 3 was located within 5 m of two other dead or dying *J. communis*. In October 2015, soil samples (approximately 300g) were collected from three points around each tree at each of seven distances (0–0.25, 0.5, 1, 2, 3, 4 and 5 m) from the main stem using a soil auger as described for site 1. The three replicate soil samples collected at each distance from the main stem were processed separately to make a total of 21 soil samples per tree. All downstream processing for real-time PCR was carried out as described for the soil sampling at site 1 except that two to four real-time PCR replicates were run per sample with each sample additionally tested using an 18S primer and probe set (Ioos, Fourrier, Iancu, & Gordon, 2009) at 2 µl DNA per qPCR as a positive control due to the low number of samples in which *P. austrocedri* DNA was amplified. Since the amounts of *P. austrocedri* DNA detected in the positive samples were very low (less than 1 pg), the unreliability of the standard curve at this level of detection meant that DNA quantification was not possible. Therefore, a positive result is reported for any sample with a Ct value of less than 38 in more than one PCR replicate per sample. Ct values above 38 were ignored because samples amplifying above this threshold produced highly unreliable technical replicates.

2.3 | Extent of infestation of *P. austrocedri* in water at site 1

For the detection of *P. austrocedri* in rainwater, six rain traps were positioned at site 1 with three traps placed at the eastern end of the site

and three traps placed at the western end, both on roughly linear transects (Figure 2). At either end, one trap was placed immediately below the start of the *J. communis* woodland at the northern boundary of the site at around 180–225 m elevation, one trap was placed within 1–3 m of symptomatic *J. communis* at 220–245 m elevation and one trap was placed approximately 5–10 m above the *J. communis* woodland at around 310–330 m elevation (Figure 2). Each rain trap collected rainwater at two positions, 0 m and 2 m above ground level. At each of these positions, rainwater was collected using a 20-cm-diameter plastic funnel containing a coarse wire mesh filter with the funnel secured using duct tape into the mouth of a 2.3-l polyethylene bottle. For the 0 m positions, bottles were placed in the ground so that the funnels sat about 10–20 cm above soil level. For the 2 m positions, each bottle was placed inside a wire mesh basket secured to a pole so that funnels sat at 2 m height. Funnels and bottles were soaked in 10% domestic bleach for a minimum of 30 min and rinsed thoroughly with tap water before use. Bottles were used for only one sampling period and disposed of. Two complete sets of funnels were used in the experiment so that a clean set could be put in place for each sampling period.

Water samples were also collected from a small river bisecting the western end of site 1 at three positions along the river: (a) approximately 100 m downstream of the *J. communis* woodland, (b) midstream as the river flowed through the woodland and (c) approximately 10 m upstream of the *J. communis* woodland (Figure 2). Additionally, water samples were collected from two seeps at each sampling date, with a seep defined as a location on the site where groundwater oozes to the surface forming a slow-flowing pool. The location of sampled seeps and quantity of seep water collected varied according to where they could be found and their state of flow at each collection date, but most were collected from close to the western end of the site. River and seep samples were collected in 2.3-L polyethylene bottles which had been cleaned before use as described for the rainwater collection.

Rain, river and seep samples were collected from site 1 on a fortnightly basis from 2 February 2017 to 17 December 2017 involving a total of six 0-m rain samples, six 2-m rain samples, three river samples and two seep samples collected at each sampling date. Additional river samples were collected in mid-January 2017 when the rain traps were set up on site. Samples were not collected between 17 December 2017 and 15 February 2018 due to a combination of seasonal holidays and bad weather preventing access to site. From 15 February 2018 until 19 April 2018, collections were made at monthly intervals, again due to bad weather or access difficulties before the last collection on 9 May 2018. For the duration of the study, total weekly precipitation was obtained from a meteorological station located at Drummond Castle, approximately 9 km from the site, and mean weekly temperature and wind velocity were obtained from a meteorological station located at Strathallan airfield, approximately 18 km from the site.

The volume of each rainwater sample was estimated before vacuum filtration through a 47-mm-diameter 3-µm pore mixed cellulose membrane filter (Merck, Darmstadt, Germany). DNA was extracted from half of each filter and the remaining half retained at −20°C. Filters were cut into small pieces and ground in 500 µl CTAB buffer (150 mM sodium phosphate, 55 mM CTAB, 1.5 M sodium chloride) with 2 × 3 mm sterile steel balls using the mixer mill MM400 (Retsch, Haan, Germany). Filters were transferred to 50-ml tubes, topped up with a further 1.5 ml CTAB buffer and incubated in a water bath at 65°C for 1 hr, vortexing every 15 min. DNA was extracted from 400 µl of the lysate using the Nucleospin Plant II Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. Each sample was collected in 50 µl elution buffer and real-time PCR carried out as described above for the investigation of radial spread in soil.

2.4 | Metabarcoding analysis of rain trap and river samples from site 1

For metabarcoding analysis of rain and river samples, 0.5 µl of each fortnightly DNA extract was pooled by month for each sampling position from February 2017 until December 2017. The ~250-bp ITS1 region was amplified from each pooled DNA sample using nested-PCR with primer pairs 18Ph2F and 5.8S-1R in the first round and ITS6 and 5.8S-1R in the second round according to the protocol of Scibetta, Schena, Chimento, Cacciola, and Cooke (2012), except that proof-reading enzyme KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) was used for the PCR to minimize errors during PCR. Second round primers were amended with overhang adapters to ensure compatibility with the Illumina index and sequencing adapters. These were as follows: forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[ITS6]andreverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[5.8S-1R] (Illumina, 2013). All *Phytophthora*-positive PCR replicates were pooled for downstream processing.

Samples were prepared for sequencing following the protocols for 16S Metagenomic Sequencing Library Preparation (Illumina, 2013) using the method as described by Riddell et al. (2019) except that instead of including a positive *Phytophthora* control mix, four samples containing a mix of synthetic sequences of known base composition were included on the plate as a check for sequence contamination across samples. Sequence data were analysed using the bioinformatic software "metapy" (https://github.com/peterthorpe5/public_scripts/tree/master/metapy) (github commit: 6fd1864) which used the sequence analysis tools Swarm (version 1.2.19) (Mahé, Rognes, Quince, De Vargas, & Dunthorn, 2014) and Bowtie (version 2.2.5) (Langmead, 2010), with sequence identity assigned using a custom-curated *Phytophthora* ITS1 database as described by Riddell et al. (2019).

3 | RESULTS

3.1 | Extent of *P. austrocedri* infestation of soil at site 1

DNA of *P. austrocedri* was detected in every soil sample collected from all three transects (Figure 3a). There was no interaction

between transect and sample position (i.e. proximity to symptomatic *J. communis* and elevation) in the amount of target DNA amplified in each sample. Transect was highly significant ($F_{2,87}$ = 11.3, p < .0001) in the model. Post hoc comparisons indicated that the amounts of DNA amplified from transect 1 were significantly lower than the amounts of DNA amplified from transects 2 and 3 (Figure 3b).

3.2 | Extent of radial spread of *P. austrocedri* from infection foci at site 2

Tree 1, which was skeletal dead and located in waterlogged soil, yielded the most *P. austrocedri*-positive soil samples from around its base, with the number of positive samples generally declining with distance from the main stem (Table 1). Tree 2, which was fully bronzed, yielded no positive samples from around its base, and tree 3, which exhibited partial dieback and a lower stem lesion, yielded only three *P. austrocedri*-positive samples which were located in no particular pattern in terms of distance from the tree (Table 1). All 21 soil samples gave good DNA amplification using the 18S primer and probe set showing that a lack of *P. austrocedri* amplification in any one sample was not due to poor DNA yields or PCR inhibition.

3.3 | Extent of infestation of *P. austrocedri* in water at site 1

A total of 442 water samples were analysed from site 1 over the duration of the study, with *P. austrocedri* DNA amplified by qPCR in only 25 samples (6%) overall. These included five 2-m rain trap samples, three 0-m rain trap samples, seven river samples and ten seep samples. Fourteen of these positive samples were collected on just two dates: 2 February 2017 (six positive samples) and 2 March 2017 (eight positive samples) (Figure 4). These included all rain trap samples yielding DNA of *P. austrocedri* across the entire study except for a single positive 2-m rain trap sample collected in July 2017 (Figure 4). No notable precipitation or wind events were recorded at the meteorological stations during each two-week sample exposure period leading up to 2 February and 2 March of that year compared with the overall weather patterns observed across the study period

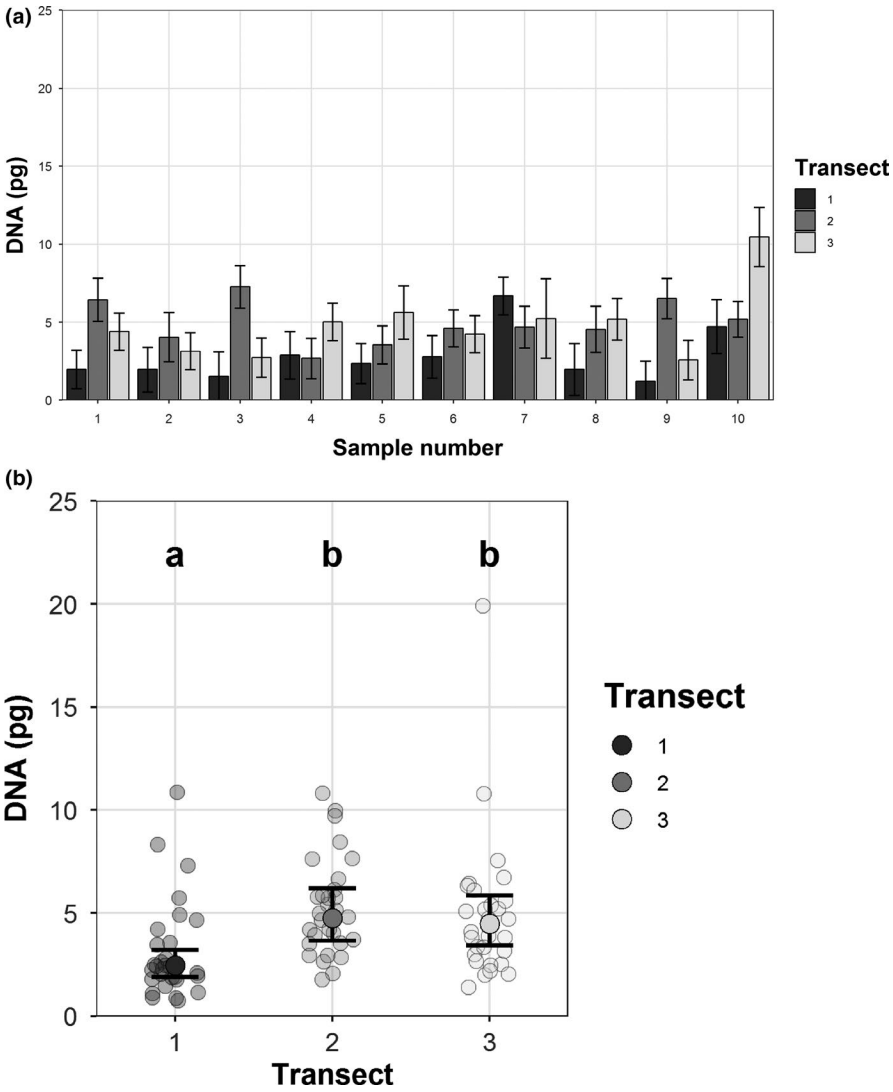

FIGURE 3 Mean quantities of *Phytophthora austrocedri* DNA (pg) amplified in soil collected from each of three transects across an infected *J. communis* woodland in Perthshire, Scotland, showing (a) means of three qPCR replicates for each of ten soil samples per transect with bars representing standard error of the mean and (b) overall mean values and back-transformed post hoc comparisons overlaid on the raw values for each sample replicate per transect. Means with the same letter at the top of the graph are not significantly different (95% confidence) using Tukey's HSD test

(Supplementary Figure S1). DNA of *P. austrocedri* was not detected in any water sample collected in April, May, September and December 2017 or in January, February and May 2018 (Figure 4). With the exception of the markedly low precipitation recorded in April 2017 (Supplementary Figure S1) when no sample yielded *P. austrocedri* DNA (Figure 4), there was no observable link between climate variables and presence/absence of *P. austrocedri* DNA in water samples.

TABLE 1 Number of soil samples in which DNA of *P. austrocedri* was amplified out of a total of three soil samples taken at each of seven distances from the main stem of symptomatic *J. communis* at an infected field site in the Cairngorm region of Scotland

Distance (m) of soil sample from main stem of *J. communis*	Number of soil samples in which DNA of *P. austrocedri* was amplified out of 3 taken at each distance		
	Tree identifier		
	1	2	3
0–0.25	3	0	0
0.5	2	0	1
1	1	0	0
2	1	0	0
3	1	0	1
4	1	0	1
5	0	0	0

Note: Tree 1 was skeletal dead, tree 2 had completely bronzed foliage and tree 3 was partially bronzed with a lesion on the lower stem.

3.4 | Metabarcoding analysis of rain trap and river samples from site 1

No *Phytophthora* sequences were detected in the four synthetic control samples, and no synthetic control sequences were detected in any environmental sample indicating an absence of sequence contamination across samples. All species were detected at a sequence abundance of 10 or higher with no species yielding reads below this level.

DNA matching fourteen *Phytophthora* species was detected in the water samples by metabarcoding, but these did not include *P. austrocedri* (Table 2). The most abundant DNA sequence matched *P. europaea/P. flexuosa/P. uliginosa* which cannot be separated due to their highly similar ITS1 sequences. This sequence was found in 0-m rain traps, 2-m rain traps and river water, with detections occurring from July to November (Table 2). The second most abundant sequence matched *P. cinnamomi*, which was found in all sample types across a time period from January to May (Table 2). DNA matching the quarantine-regulated pathogen *P. ramorum* was detected in a 0-m rain trap sample collected in March, a 2-m rain trap sample collected in May and in a river sample collected in August (Table 2). Two 0-m rain trap samples collected in April yielded DNA matching another quarantine-regulated pathogen, *P. kernoviae* (Table 2). Other species detected infrequently, and only in rain traps, were *P. cambivora*, *P. foliorum*, *P. obscura* and *P. sojae* (Table 2). DNA matching *P. cactorum* was detected in two samples: one river and one rain trap (Table 2). *Phytophthora* species detected only in river samples included *P. gibbosa*, *P. gonapodyides*, *P. pseudosyringae*, *P. syringae* and *P.* taxon *paludosa* (Table 2). No sample yielded *Phytophthora* DNA in December (Table 2).

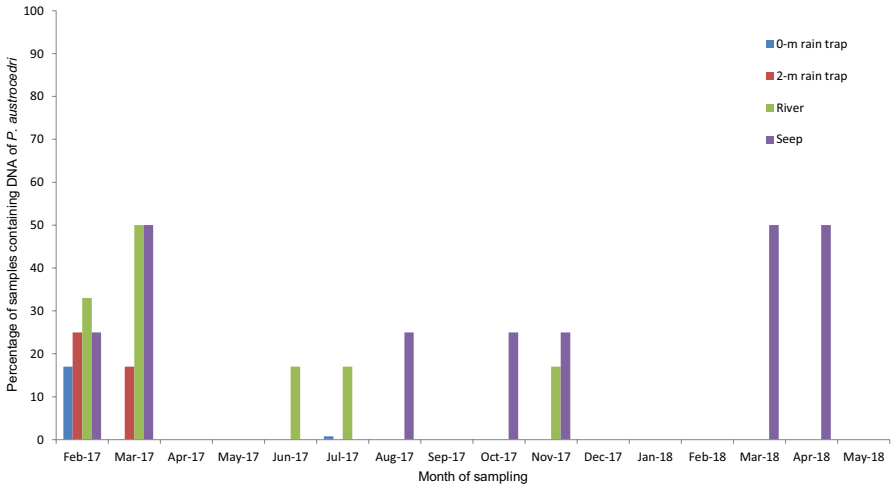

FIGURE 4 Detection of *P. austrocedri* in water samples collected from a naturally infected *J. communis* woodland in Perthshire, Scotland. Data show the percentage of samples in which *P. austrocedri* DNA was detected using qPCR during fortnightly sampling periods from February 2017 to May 2018. Data represent samples collected from rain traps located at heights of 0m (n = 12) and 2m (n = 12), natural seeps (n = 4) and a river (n = 6) with data pooled by month (where n = total number of samples of each type collected per month). Exceptions to the number of samples collected per month occurred when poor weather prevented access to the site, as a result of which no samples were collected between mid-December 2017 and mid-February 2018, and samples were collected monthly rather than fortnightly between mid-February 2018 and mid-April 2018. Only two seep samples were collected per month in March and April 2018

TABLE 2 *Phytophthora* species detected in 0-m rain traps, 2-m rain traps and an adjacent river, all located at an upland *J. communis* site in Perthshire, Scotland, and with sampling conducted from February to December 2017 (rain traps) and from January to December 2017 (river)

	Number of samples in which each species found per month/ total number of Illumina sequence reads											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
0-m rain trap												
P. cinnamomi	NS		3/79,883	1/32,308	3/34,442							
P. europaea/flexuosa/ uliginosa	NS							3/37,194	3/53,908	1/10,356		
P. kernoviae	NS			2/82,889								
P. foliorum	NS		1/25,978									
P. sojae	NS						1/8,117					
P. cactorum	NS						1/1,159					
P. obscura	NS		1/1,099									
P. ramorum	NS		1/71									
2-m rain trap												
P. europaea/flexuosa/ uliginosa	NS						1/95	1/52,037	3/52,048	2/20,864	2/14,213	
P. cinnamomi	NS	1/55,704	2/58,053		3/102,135							
P. obscura	NS	1/28,370					2/268					
P. ramorum	NS				2/1,164							
P. cambivora	NS			1/38,837								
River												
P. pseudosyringae						1/29,970		2/21,998	1/14,508	1/3,860		
P. gonapodyides	1/41,891					1/19,650			2/2,568			
P. cinnamomi	2/87,255			1/4,384								
P. syringae			1/8,737					2/10,577				
P. europaea/flexuosa/ uliginosa								2/6,982				
P. cactorum								1/5,916				
P. ramorum								1/2,559				
P. gibbosa								1/2,445				
P. taxon paludosa									1/568			

Note: Data represent the number of samples in which each species was found per month and the total number of Illumina sequence reads per species per month. Species are listed in order of sample abundance and then sequence read abundance.

Abbreviations: NS, not sampled.

4 | DISCUSSION

Investigations of radial spread found that *P. austrocedri* was only consistently detected in soil samples collected from around a long-dead *J. communis* in conditions of high soil moisture. The decreasing frequency of detection of the pathogen with increasing distance from the dead *J. communis* suggests a slow, natural spread of the pathogen in soil, requiring high moisture conditions. The fact that *P. austrocedri* was detected in all soil samples collected from transects across a heavily infected *J. communis* woodland, regardless of elevation or proximity to symptomatic *J. communis*, suggests that other pathways have aided *P. austrocedri* dissemination at this site. Elliot et al. (2015) consistently amplified DNA of *P. austrocedri* in soil from footpaths and animal tracks at eight different infected *J. communis* woodlands as well as from boots after walking through one of the sites. It is likely that animals carrying infected soil and root debris could have vectored further spread at site 1 which is ranged by deer, sheep-grazed and formerly cattle-grazed. The finding that less *P. austrocedri* DNA was amplified from soil on the easternmost transect fits with a possible later spread of the pathogen into the predominantly drier, eastern part of the site, as indicated by the presence of fewer long-dead trees. Site 2, which is ranged by deer and regularly sheep-grazed in one section, forms part of a continuous *J. communis*, *Pinus* and *Betula* habitat within the Caledonian pine forest which extends across the lower Cairngorms. It is likely that *P. austrocedri* will continue to spread *via* animal (and/or human)-vectored movement of infested soil debris throughout this region which is one of the most important habitats for biodiversity conservation in Scotland. Ecological modelling aimed at better understanding how factors such as soil moisture, hydrology, slope, vegetation and *J. communis* connectivity contribute to pathogen impact is being carried out (F. Donald, University of Cambridge, unpublished) to enable less vulnerable sites to be identified and targeted for conservation measures.

qPCR analysis of rain, river and seep samples over a fifteen-month period at site 1 yielded a very low frequency of detection of *P. austrocedri* in water. DNA of *P. austrocedri* was detected in only five 2-m rain trap samples over this period, in February and March only. Although these findings fit with the potential seasonal activity of a cool-temperature pathogen like *P. austrocedri* (Henricot et al., 2017), it is nonetheless unconvincing evidence of the sort of effective aerial dispersal required for concurrent infections of geographically distant sites. It is possible that these positive findings in the 2-m rain traps may have been the result of bird-vectored transmission. The pathogen was also occasionally detected in river water and most frequently in seeps, which tend to have a higher soil sediment content. It is therefore surmised that waterborne spread of *P. austrocedri* is restricted to within-site, primarily via percolation of water through soil and into streams, with local flooding events likely to result in episodic dispersal of inoculum along flatter terrain features, hence the strong observed association of *J. communis* dieback and mortality alongside watercourses and in patches within wet flushes and on lower, flatter ground (Green et al., 2015).

Metabarcoding of monthly pooled rain trap and river samples did not detect *P. austrocedri* but did enable the detection of fourteen other *Phytophthora* species, including the five species also detected in soil at the site by Riddell et al. (2019). Both caducous and non-caducous species were detected in upper and lower rainfall traps and in the river, and there were occasional detections of quarantine-regulated species. These included *P. ramorum*, which is causing extensive damage to *Larix kaempferi* in predominantly western parts of the UK (Green & Webber, 2012), and *P. kernoviae*, which infects *Vaccinium* (Beales, Giltrap, Payne, & Ingram, 2009) and some woody hosts. A species very closely related to *P. ramorum*, *P. foliorum*, was also detected in a single rain trap sample. *Phytophthora foliorum* was first described from nursery-grown Azalea plants in the United States (Donahoo et al., 2006) and in Spain (Jung et al., 2016), and has only been recently recorded in the UK on Rhododendron in north-west Scotland (Schlenzig, Purser, & Perez-Sierra, 2016). Its wider distribution and host range in the UK is unknown.

The most abundant DNA sequence matched *P. europaea/P. flexuosa/P. uliginosa*, which cannot be distinguished based on their ITS1 sequences. DNA matching these species has been found previously in Scotland (Riddell et al., 2019), but the associated organism has never been cultured in the UK. Interestingly, the second most abundant *Phytophthora* species detected was *P. cinnamomi* which predominated in winter and spring-collected water samples. This pathogen is non-caducous, has a relatively high optimum temperature for growth and a very broad global host range encompassing many woody species including *Juniperus* spp. It was recently isolated from declining *Juniperus oxycedrus* in the Mediterranean region (Scanu, Linaldeddu, Deidda, & Jung, 2015) but has never been isolated from *J. communis* in the UK despite the sampling of a large number of trees (Green et al., 2015).

Other woody-host infecting Phytophthoras detected in the rainfall traps at various times over the course of the experiment included *P. cactorum* and *P. cambivora*. Since DNA of the latter species has been detected previously in lesions of *J. communis* at the site (S. Seddaiu, Forest Research, UK, unpublished data), there is a question as to the extent to which it is contributing to dieback symptoms. Again, neither this pathogen nor any other *Phytophthora* species detected here by metabarcoding has been isolated into culture from lesions on *J. communis* in the UK. The closest known related species to *P. austrocedri*, *P. obscura*, was also detected in a few rain trap samples. This species was first described in 2012 (Grünwald, Werres, Goss, Taylor, & Fieland, 2012) but is not known to cause significant damage to any host.

Of the species detected in river samples only, *P. gonapodyides* is a ubiquitous clade 6 species which flourishes in aquatic habitats and is thought to play a role in breakdown of plant debris (Brasier, Cooke, Duncan, & Hansen, 2003); *P. pseudosyringae* has been found frequently in Britain infecting *Nothofagus* spp., *Fagus sylvatica* (Scanu & Webber, 2016), *Larix kaempferi* (J. Webber and A. Harris, Forest Research, UK, personal communication) and *Vaccinium myrtillus* (Beales, Giltrap, Webb, & Ozolina, 2009); *P. syringae* is considered common in Britain causing disease on a wide range of woody and

non-woody hosts (Cooke, 2015); and *P. gibbosa* and *P.* taxon *paludosa* are clade 6 species recently described from waterways in natural ecosystems in Australia (Jung et al., 2011) and have not previously been reported in Britain.

The lack of metabarcoding amplification of *P. austrocedri* in any water sample is at odds with qPCR analysis of the same samples. Using the same metabarcoding method, reference database and bioinformatic pipeline, Riddell et al. (2019) amplified abundant reads of *P. austrocedri* in soil samples collected from the site so it is highly unlikely to be due to a *P. austrocedri*-specific sequencing issue. Since qPCR was conducted on individual DNA samples and metabarcoding analyses done on two pooled DNA samples, the latter may have been too dilute to enable nested-PCR amplification of *P. austrocedri*. It would be useful to assess the relative sensitivity of both methods in environmental samples. It is to be noted too that seep samples, which generally yielded more *P. austrocedri*-positive qPCR samples than other water samples, were not processed for metabarcoding in this study; a retrospective metabarcoding analysis of these samples might reveal the pathogen.

Another point to raise concerning the metabarcoding of rain samples was that many of these samples contained a very high proportion of DNA from downy mildew species of genus *Peronospora*, *Hyaloperonospora* and *Bremia* (data not shown) which cross-react with the PCR primers. Collecting the traps on a weekly rather than fortnightly basis may have reduced the level of downy mildews present in the samples. Weekly sampling, however, was not feasible for this experiment due to the time required to access the site and prepare and process the materials. The finding of non-caducous *Phytophthora* species, notably *P. cinnamomi*, in the 2-m rain traps which were sited high enough to avoid rain splash from the ground, suggests some form of aerial vectoring. The frequent observation of bird droppings caught in the funnel mesh of the upper rain traps suggests that birds perched on them. Thus, some form of bird-aided transmission remains a possibility which might also explain the occasional qPCR detection of *P. austrocedri* in the 2-m rain traps.

This study's findings suggest that natural spread of *P. austrocedri* is most likely limited to a "within-site" distribution via soil water run-off, aided by animal and human activity and possibly the occasional bird-vectored transmission of infested soil and plant debris. Thus, the question remains as to how a single genotype of the pathogen (Henricot et al., 2017), signifying fairly recent arrival, has come to infect *J. communis* across such a wide geographical distribution in Britain. Prior to the epidemic of *P. austrocedri*, there were concerns over a general decline of *J. communis* populations in Britain occurring over the last seventy years due to overgrazing, burning and lack of regeneration (Preston et al., 2002; Thomas et al., 2007). These concerns prompted an acceleration of conservation plantings across the country from around the late 1990s onwards, aimed at bolstering locally declining *J. communis* woodlands. These programmes used propagation methods such as those outlined by Broome (2003) and Plantlife (2005) whereby seed collected from local populations are raised, in some cases by commercial plant nurseries trading in other *Juniperus* and Cupressaceae hosts, before being planted back out onto the site. Numerous *P. austrocedri*-infected *J. communis* woodlands in the Cumbria, Yorkshire, County Durham, and across Scotland were subject to supplementary planting of this sort (Green et al., 2015). Given the findings of *P. austrocedri* in UK plant nurseries, as outlined earlier, the planting out of infected *J. communis* is clearly a potential pathway of introduction of the pathogen into vulnerable sites. Therefore, we suggest that a single genotype of *P. austrocedri* circulating in traded *J. communis* and present in commercial nurseries may have been inadvertently introduced into wild *J. communis* populations in Britain through restoration plantings. A gathering body of evidence is demonstrating the introduction and spread of invasive *Phytophthora* species in California wildlands as a result of restoration schemes involving planted native species raised in contaminated nurseries (Garbelotto, Frankel, & Scanu, 2018). More evidence needs to be gathered on the potential for spread of *Phytophthora* through restoration plantings in the UK, including an assessment of *Phytophthora* infestations in plant nurseries growing restoration stock and a mapping of the distribution of disease outbreaks in relation to plantings. Until more information is available, it would be wise to implement stringent biosecurity practices when raising native stock destined for planting onto ecologically sensitive sites.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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