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Assessment of Acute Oak Decline in Latvia

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Abstract

Acute Oak Decline (AOD) is a complex disease affecting oaks (*Quercus* spp.). Typical symptoms include a dark, sticky exudate from bark cracks. Larval galleries of the beetle *Agrilus biguttatus* under the bark of affected trees are often reported. AOD is characterized by oak decline, after which a large proportion of the oaks die, but some survive. In Latvia, oaks with AOD symptoms were first observed in 2017, and the presence of *Gibbsiella quercinecans* and *Brenneria goodwinii* in the bark exudate was confirmed. A country-wide survey was initiated to assess the prevalence of oaks with AOD symptoms. In addition, experimental plots were established to monitor the progression of AOD in infected sites. A total of 1,329 forest stands were surveyed; 111 exudate samples were collected and 36 samples were positive for one of both *B. goodwinii* and *G. quercinecans*. Larval galleries or other signs of the beetle *A. biguttatus* were not found in association with AOD symptoms. Four trial plots were established and routinely monitored over a four-year period for AOD symptoms, presence of *G. quercinecans* and *B. goodwinii*, and an assessment of fungal species was done. In the trial plots, AOD symptoms peaked in 2019. Presence of the bacteria was not detected in all exudate samples collected regularly over summer from the trial plots. The presence of the bacteria was confirmed only in a few soil samples collected along a transect in one trial plot. One or both bacteria were identified in a few soil and wood samples collected from asymptomatic oak stands. Assessment of fungal species in both symptomatic and healthy trees did not identify significant differences in species composition, soil pH, fine root vitality, and mycorrhization. Environmental factor analysis indicated that precipitation in the previous autumn is related to a higher detection possibility of *G. quercinecans* and *B. goodwinii* in exudate samples collected in the following year. Continued monitoring for AOD symptoms, focussing on mature oak trees is required to identify future potential AOD outbreaks in Latvia.

Keywords: *Quercus robur*; AOD; monitoring; qPCR; phytopathology; environmental factors

Introduction

Acute Oak Decline (AOD) is a complex disease (influenced by both biological and abiotic factors) affecting oaks (*Quercus* spp.). The symptoms include a dark exudate from bark cracks and lesions, crown dieback and larval galleries of the beetle *Agrilus biguttatus* under the bark (Denman et al. 2014). Crown condition is also indicative of oak decline (Finch et al. 2021).

AOD is characterized by a five to ten-year period of oak decline, after which a large proportion of the oaks die, but some survive and their condition stabilizes, and symptoms may disappear completely (Denman and Webber 2009). Forest decline, as commonly described, results from various factors, both non-living (abiotic) and living (biotic), that weaken the overall health of trees. This decline initiates with predisposing factors, which weaken the tree's ability to resist damage and make it more vulnerable to harmful living agents, eventually causing the death of the tree (Marçais and Bréda 2006). Oaks affected by AOD are characterized by long cracks (5–10 cm) in the bark of the trunk, which exude a dark, sticky substrate. Initially, under the bark, around the crack, necrotic tissue is observed, which develops in cavities filled with exudate. In the spring, when the bark ruptures, the exudate flows out through the gaps between the bark plates. In warmer weather, the exudate may dry out to form shiny, sticky drops at the bark fractures. It has been observed that trees with typical symptoms die within 3–5 years (Denman and Webber 2009). It is still unclear whether the tree dies due to an increase in the proportion of necrotic tissue (Maddock et al. 2023), disruption of the vascular system, or due to secondary factors, such as the activity of *A. biguttatus* (Denman and Webber 2009).

Comparison of bacterial and fungal diversity in typical AOD symptomatic and asymptomatic oak tissues, indicate that the most common bacterial species from symptomatic tissues are *Gibbsiella quercinecans*, *Brenneria goodwinii* and *Rahnella victoriana* (Brady et al. 2017) and inoculation with *B. goodwinii* and *G. quercinecans*

induces tissue necrosis in oak logs and trees, and together with the beetle *A. biguttatus*, can result in symptoms characteristic of AOD (Denman et al. 2018). It has been reported that *B. goodwinii* cannot survive in rainwater and forest soil and for long periods outside the host plant, but *G. quercinecans* has been shown to survive in rainwater for at least 84 days and in forest soils for at least 28 days (Pettifor et al. 2020). However, according to recent evidence, the bacteria associated with AOD did not show differences between tree health categories but did exhibit variations among different sites. The authors suggest that these AOD-associated bacteria may actually be part of the normal oak microbiome (Gathercole et al. 2021).

AOD was first described in Britain (Denman and Webber 2009), but has also been reported in Iran (Moradi-Amirabad et al. 2019), Switzerland (Ruffner et al. 2020), Latvia (Zalkalns and Celma 2021), Poland (Tkaczyk et al. 2021), Portugal (Fernandes et al. 2022) and Slovakia (Tkaczyk et al. 2024). The distribution of AOD has also been correlated with environmental factors in the United Kingdom (Brown et al. 2018), and the presence of oaks with AOD symptoms is increased in areas with lower precipitation, higher temperatures, and at lower elevations. In general, oak decline can be influenced by many factors, and the most common disorders of oak growth and diseases are moderated by prolonged exposure to abiotic factors, such as excessive drought or, conversely, moisture. These conditions can reduce the natural defences of trees and therefore they can be more susceptible to various pathogens. The environmental factors that influence oak decline processes can vary according to location, and the intensity of symptoms is dependent on both abiotic and biotic factors (Marçais and Bréda 2006).

Pedunculate oak (*Quercus robur* L.) is the only autochthonous oak species in Latvia and is distributed throughout the country. In 2023, *Q. robur* dominated stands covered a total area of 10,332 hectares, with almost half of this area found in the western region of Latvia (Kurzeme – 4,893 hectares) (O. Zaļkalns, pers. comm.). In Latvia, oaks with AOD symptoms were first observed in 2017 in the Talsi Hills Forest, western Latvia. Samples of the bark exudate were analysed by colleagues at Forest Research in the UK, confirming the presence of *G. quercinecans* and *B. goodwinii* in the exudate (G. Bokuma, pers. comm.). At the end of 2021, a study was initiated to develop solutions and recommendations for the management of AOD in the Talsi Hills Forest Research Station, which could then be applied to the rest of Latvia as required. The main tasks of the study were to determine the prevalence of the disease throughout Latvia by conducting forest stand surveys, analysis of AOD symptoms, influencing factors and dynamics, and development of recommendations for AOD containment and management of oak stands in Latvia. Within this study, a country-wide survey was initiated to assess the prevalence of oaks with AOD symptoms. In addition, experimental plots were established in sites where AOD had been confirmed by the presence of *G. quercinecans* and *B. goodwinii* in exudates collected from symptomatic trees to monitor the progression of AOD in the infected sites.

Materials and methods

Study area

Climatic conditions in Latvia are influenced by the transport of cyclonic air masses from the Atlantic Ocean, topography, distance to the Baltic Sea, and coverage of forests and mires. Therefore, the atmospheric humidity is relatively high and precipitation distribution throughout the year is uneven. The average annual temperature is +7.5 to +7.9°C near the coast of the Baltic Sea and +5.7°C in the eastern part (Alūksne and Vidzeme uplands). The warmest month in the year is July with an average temperature of +17.8°C, but the coldest is February with an average temperature of –3.1°C. The average annual mean precipitation is 685.6 mm. July and August are the wettest months, with an average precipitation level of 76.8 and 75.7 mm, respectively, while April is the driest month in the year, with an average precipitation level of 35.8 mm. The annual average mean relative humidity in Latvia is 81%. The lowest relative humidity is in the spring season, in May, when the average air humidity level is 71%, but the highest is in the winter season in December, 89% (LVGMC 2024).

Oak (*Q. robur*) stands are found in various soil types, but most often in dry soils. Oak stands in Latvia are widely distributed, most often these are small stands or old planted gardens with some large oak trees near old houses (Ikauniece 2017).

Oak stand selection

Stands, where the dominant species was oak, were selected from the State Forest Service State Forest Register, using the criteria: stand age, at least 50 years; stand composition (forest stand formula Oz), i.e. 4, 5, 6, and 10 oaks, corresponding 40 to 100% of oak by stock volume, and stand area, at least 1 ha.

A total of 1,329 forest stands were surveyed, and if typical AOD symptoms were detected, then the exudate from the lesion was collected using swabs and sent to the National Phytosanitary Laboratory for molecular analysis. The exudate swabs were processed and analysed for the presence of *Brenneria goodwinii* and *Gibbsiella quercinecans* as described previously (Zalkalns and Celma 2021), following the methodology developed by Forest Research in the UK (Crampton et al. 2020). Molecular analysis was performed using qPCR, utilizing a Rotor-Gene Q thermocycler (Qiagen). For the *G. quercinecans* assay, a specific primer pair targeting the rpo gene (GQ gyrB qPCR F / GQ gyrB R) (Pettifor et al. 2020) was used and subsequent melting curve analysis was performed to identify specific melt peaks. For the *B. goodwinii* assay targeting the gyrB

gene, a primer pair (Bg99F/Bg179R previously described as BG-F/BG-R) and hydrolysis probe (Bg124P previously described as BG-P) (Crampton et al. 2020) were used. A more detailed description of the qPCR assays was published previously (Zalkalns and Celma 2021).

Establishment of trial plots and visual assessment of oak individuals

Four trial plots – Šķēde 1, Šķēde 2, Cīrava and Kazdanga (Figure 1) – were established in the western region of Latvia. Circular plots (2,000 m^2, r = 25.23 m) were laid down in all sites except Kazdanga, where a circular plot was not able to be defined due to the shape of the oak stand. The circular plots were divided into three concentric sectors. In sector A (the entire plot), all standing (alive and dead) oak trees with a DBH of > 10 cm were measured. In sector B (1,000 m^2, r = 17.84 m), trees of all species with a DBH > 10 cm were measured. In sector C (200 m^2, r = 7.98 m), trees of all species with a DBH > 6 cm were measured.

Each measured tree was numbered, and a laminated number was fixed to the trunk. The azimuth (0.5° accuracy), and the distance (1.0 cm accuracy) from the centre of the plot was measured for each numbered tree. The species of each tree was recorded, along with DBH and height (0.1 cm accuracy), biotic and abiotic damages. Oak trees were repeatedly scored for the typical visual AOD symptoms according to the scale:

0 – no visible symptoms;
1 – presence of old bark cracks with no visible exudate;
2 – one bark crack with fresh exudate;
3 – two or three bark cracks with fresh exudate;
4 – three or more bark cracks with fresh exudate.

The interpolation map of AOD spread in the sample plots was derived using b-spline interpolation in the QGIS application, Multilevel B-Spline procedure SAGA (System for Automated Geoscientific Analyses) (Lee et al. 1997, Conrad 2006). The effect of tree trunk diameter, sample plot location and calendar year on the frequency of visual AOD symptoms was analysed using

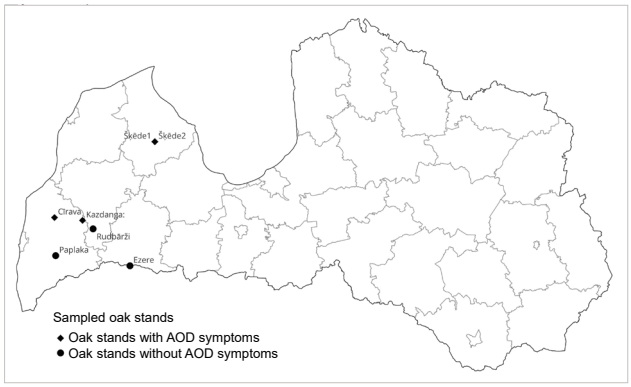

Figure 1. Location of trial plots: oak stands with and without AOD symptoms

Ordinal Logistic Regression (polr procedure in MASS package, R statistics) (Parry 2020). The following model was used:

Model = polr (Symptoms~Year × SP + D), where

Symptoms – corresponds to visual AOD symptoms, classified in 5 classes: "No symptoms", "Old (healed) scars", "Fresh scar", "Several fresh scars", "Multiple fresh scars";
Year – corresponds to calendar year;
SP – corresponds to sample plot;
D – corresponds to tree trunk diameter at the height of 1.3 metres.

Collection and analysis of plant and soil samples from trial plots

Wood and bark samples were collected from all oak trees (alive and dead) in the plots using an increment borer. Soil samples (0 to 10 cm depth) were collected using cylinders (height = 10 cm, diameter = 5 cm). Samples were stored at +4°C before DNA extraction, not longer than 72 h, if DNA was not extracted within this time, the samples were frozen at –20°C. DNA was extracted from wood and bark samples using a modified CTAB protocol (Porebski et al. 1997), and from soil samples using the DNeasy PowerSoil Kit (Qiagen). DNA samples were analysed for the presence of *B. goodwinii* and *G. quercinecans* in the Molecular Genetic Laboratory of LSFRI 'Silava' using the same real-time qPCR protocol as in the National Phytosanitary Laboratory (Zalkalns and Celma 2021).

The Šķēde 1 and Šķēde 2 plots were also repeatedly surveyed for bark cracks and exudate in 2021 (six times – 21/05, 04/06, 09/06, 15/06, 21/06, 02/07). If exudate was found, samples were taken and analysed in the Molecular Genetic Laboratory of LSFRI 'Silava' as previously described.

In 2021, soil and wood samples were collected in three oak stands (Paplaka, Rudbārži and Ezere) (Figure 1), where no oak individuals with visual signs of AOD (bark lesions with exudate) were identified. Wood and bark samples from eight oak individuals in each stand were collected with an increment borer, and soil samples were collected immediately next to each sampled tree, as described previously. DNA was extracted and analysed in the Molecular Genetic Laboratory of LSFRI 'Silava' as described previously.

Collection of phytopathological samples and analysis

Wood samples were taken at the stem base from all oak trees in the plots with bark lesions with exudate, and a similar number of asymptomatic trees. In total, 33 asymptomatic and 33 symptomatic trees were examined. In addition, to identify any potential pathogens that could be source of secondary infection at the site of the lesions, wood core samples were collected from symptomatic oak trees from the site of bark cracks with exudate approxi-

mately 1–2 cm below lesions (one lesion per tree). An increment borer sterilized with 70% ethanol before each sampling was used to collect samples. Immediately after the sampling, wood cores were individually placed into sterile plastic tubes. Wood samples were stored at +4°C before laboratory processing. In the laboratory, wood cores were flame surface sterilized and placed onto malt agar media in Petri plates. Samples were incubated at room temperature and inspected every 2–3 days for mycelial growth. Pure mycelial isolates were grown on separate Petri plates, examined under the microscope, and grouped according to mycelial morphotype. Four genera were identified microscopically: *Cladosporium* spp., *Mucor* spp., *Penicillium* spp. and *Trichoderma* spp. One isolate from the remaining morphotypes was used for species identification by sequencing the ITS region using ITS1F and ITS4 PCR primers.

In the trial plots, both oak individuals and ground cover were surveyed for the occurrence of fruiting bodies. Fruiting bodies were collected and the taxa were identified morphologically.

Soil and fine root samples were collected from the monitoring plots using the soil core cylinders described above. Samples were collected 1 m from oak trees in three replicates. Collected samples from each tree were pooled and analysed as one bulk sample. A similar number of visually healthy and symptomatic individuals were sampled in each plot. A total of 40 oaks were sampled (20 visually healthy, and 20 symptomatic). All woody roots were picked from soil samples and rinsed under tap water. The vitality of fine roots was examined under a stereomicroscope in 30 sight fields for each sample. Mycorrhiza types were assessed based on the methodology developed by Agerer (1987–2002). Soil pH was determined using KCl extraction.

Correlation of Gibbsiella quercinecans and Brenneria goodwinii detection with meteorological factors

Information about the exudate samples collected throughout the territory of Latvia and analysed in the National Phytosanitary Laboratory was collected in *Microsoft Excel* spreadsheets: analysis results, sample collection date, sample receipt date at the laboratory, analysis date, and location of sampling plae. Also, meteorological information was collected from State LLC Latvian Environment Geology and Meteorology Centre (LVGMC) database (LVGMC 2024): information about total daily precipitation (mm), average temperature (°C) and relative air humidity (%) in the sampling area on the sampling date.

The obtained data was divided and analysed in several stages starting from the sampling date. Using *Microsoft Excel* spreadsheets, a descriptive statistics analysis was done to get the median of the data set. Calculations were performed for each period for every sample and the results were added to the main database. Median of data was used because data has extreme values, outliers (Figure 2).

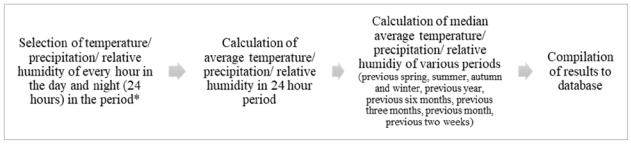

Figure 2. Data analysis and collection process

* Data from the database "Latvian Environment, Geology and Meteorology Centre".

Exudate samples were defined as positive if a positive PCR result was obtained for one or both bacteria (*G. quercinecans* and *B. goodwinii*) and negative if it was negative for both bacteria, despite the sample was collected from the oak having typical AOD visual symptoms. Using random data selection, two groups (positive and negative) with 36 samples in each one were constructed.

The RStudio software package (RStudio Team 2020) was employed to test data for normality using the Shapiro-Wilk test. The Wilcoxon Rank-Sum test was applied to evaluate differences in metrological factors between the two groups (positive and negative). Kendall's rank correlation was used to estimate the relationship between sample groups (positive or negative) and meteorological data. It was concluded that the correlation is very strong if Tau > 0.5, and moderate if Tau is between 0.3 and 0.5, weak if Tau value is less than 0.3 (Akoglu 2018).

Results

Survey of oaks stands throughout Latvia

In the 1,329 forest stands surveyed, 111 exudate samples were collected and analysed for the presence of *B. goodwinii* and *G. quercinecans* in the National Phytosanitary Laboratory. As a result, 36 samples were positive for one or both bacteria, but 75 were negative (Zalkalns and Celma 2021) (Figure 3).

Of the 111 collected exudate samples, 11 were collected in spring (March–May), of which 4 were positive for one or both bacteria, 35 were collected in summer (June–

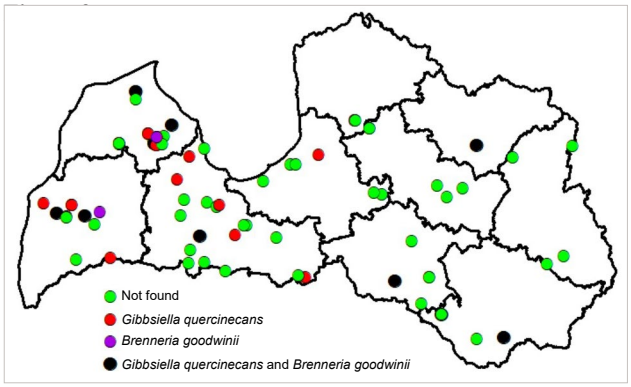

Figure 3. Location of collected exudate samples and results of qPCR analyses (adapted from Zalkalns and Celma 2021). Due to map scale, one point may represent more than one collected exudate sample

August), of which 5 were positive, and 65 were collected in autumn (September–November), of which 27 were positive (Figure 4).

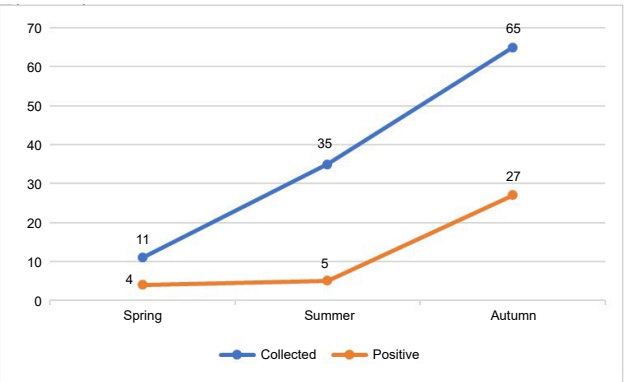

Figure 4. Number of samples collected and number of samples positive for one or both bacteria, by season

Survey of oaks in trial plots 2018–2021

Assessment of visual AOD symptoms on oak trees in the trial plots was done annually between 2018 and 2021. The number of individuals with fresh exudate in bark cracks was highest in 2019, and then declined, with the number of individuals with old bark cracks with no visible exudate correspondingly increasing (Figure 5).

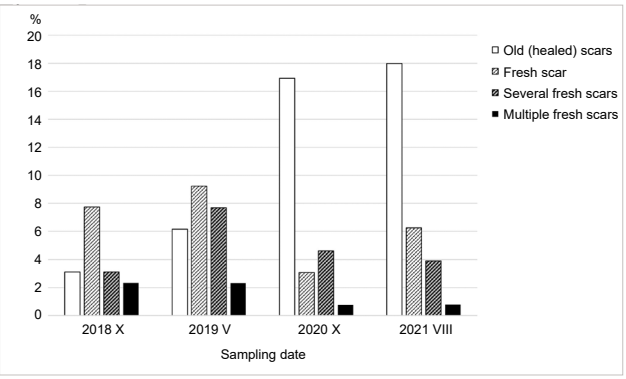

Figure 5. Number of oak individuals in AOD symptom categories 1–4 in the trial plots between 2018 and 2021

There were significant differences in frequencies of AOD symptoms among sample plots, as well as a significant correlation between AOD symptoms and tree diameter, but variation among years was not significant (Table 1).

Table 1. Analysis of frequencies of AOD symptoms in sample plots (ANOVA (model))

	LR Chisq	Df	Pr(> Chisq)
Year	1.375	1	0.24100
Sample Plot	70.467	3	3.39e-15 ***
Tree Diameter	4.855	1	0.02757 *
Year: Sample Plot	0.208	3	0.97636

* $P \leq 0.05$, *** $P \leq 0.001$.

A spatial analysis of oak trees according to visual AOD symptom class was done in the Šķēde 1 and Šķēde 2 trial plots. Individuals with visual AOD symptoms were spatially clustered (Figure 6). As described previously, the visual AOD symptoms peaked in 2019, and newly deceased oak individuals were found in both trial plots in the spring of 2019. The spatial spread of visual AOD symptoms was not significantly different between years.

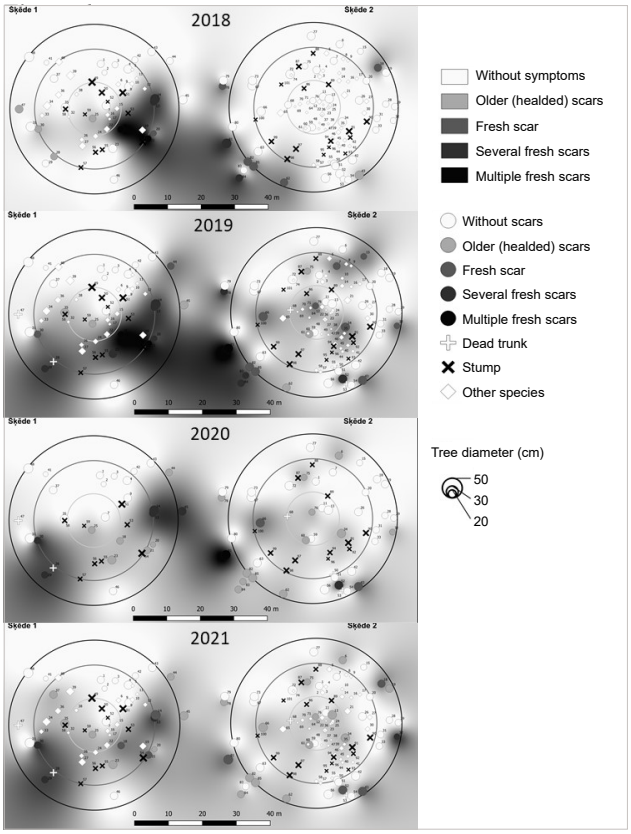

Figure 6. Spatial distribution of oak trees according to visual AOD symptom scores (circles) and interpolation map of AOD spread (shaded areas) in the Šķēde 1 and Šķēde 2 plots 2018–2021 (b-spline interpolation SAGA)

Trees with larger trunk diameters also had a higher frequency of visual AOD symptoms (Figure 7).

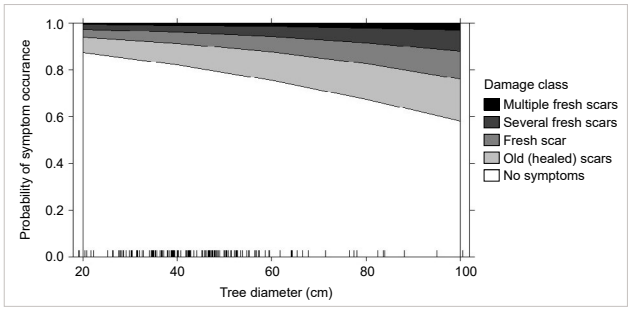

Figure 7. Tree diameter (DBH) and probability of AOD symptom occurrence according to the classes. Lines on *x* axis represent individual trees

The presence of *Agrilus biguttatus*, either live mature individuals or larvae, or larval tunnels and exit holes in the bark, was not detected in the four trial plots or in any other of the surveyed oak stands.

Detection of the bacteria Brenneria goodwinii and Gibbsiella quercinecans in the trial plots

Wood samples were collected from living and dead oak individuals in the sample plots. A total of 95 oak individuals were analysed, and *B. goodwinii* was detected in seven samples, while *G. quercinecans* was not detected in any of the samples. In the Cīrava and Kazdanga plots, neither *B. goodwinii* or *G. quercinecans* were detected in any of the analysed wood samples (from a total of 26 oak individuals, of which seven had stem cracks with exudate, and 19 were without visual symptoms). In the Šķēde 1 plot, wood samples from 36 oak individuals were analysed (13 with symptoms, 14 without, 3 standing dead trees, 6 stumps). Of these, *B. goodwinii* was detected in six samples (1 individual with symptoms, 4 individuals without symptoms, 1 stump). *G. quercinecans* was not detected in any of the samples. In the Šķēde 2 plot, wood samples from 33 oak individuals were analysed (14 with symptoms, 14 without, 1 standing dead tree, 4 stumps). Of these, *B. goodwinii* was detected in one sample (1 individual with symptoms). *G. quercinecans* was not detected in any of the samples.

From May to July 2021, a more frequent (approximately every two weeks) visual assessment of AOD symptoms in the Šķēde 1 and Šķēde 2 plots was done. This assessment did not reveal a large number of individuals with fresh exudate, and no rapid development of AOD symptoms was observed. This is probably a reflection of the lower incidence of AOD symptoms in all plots in 2021 (Figure 4). A total of 21 exudate swabs were collected from the two plots over the six assessment time points. Analysis by qPCR indicated that only three samples were positive for one or both bacteria. Both bacteria were found in one exudate sample (collected in the Šķēde 2 plot on 9.06.2021), and only *B. goodwinii* was found in two samples, collected in the Šķēde 2 plot on 9.06.2021 and 15.06.2021). The positive samples collected on 9.06.2021 were collected from two different bark lesions on one individual oak tree.

In the Šķēde 1 plot, soil samples were collected in a transect along the diameter of the plot. The distance of the soil sampling site to the closest oak tree was not determined. A total of 25 soil samples were collected, with approximately 1 m between each sample. *B. goodwinii* was detected in one soil sample, *G. quercinecans* in five, and both bacteria in two samples. This indicates that despite the long-term presence of AOD symptoms in this stand (the presence of *B. goodwinii* and *G. quercinecans* was confirmed in 2017), these bacteria are not widespread in the soil of the affected stand.

In 2021, soil and wood samples were collected in three oak stands (Paplaka, Rudbārži and Ezere), where no oak individuals with visual signs of AOD (bark lesions with exudate) were identified. Wood samples were collected from eight individuals in each stand, as well as soil samples from the base of the sampled individual (eight soil samples from Paplaka and Ezere, six soil samples from Rudbārži, as the sampled individuals were growing close to each other). *B. goodwinii* was detected in only one wood sample from Rudbārži, *G. quercinecans* was not detected in any of the samples. *G. quercinecans* was detected in 13 soil samples (4 from Ezere, 7 from Paplaka, and 2 from Rudbārži). *B. goodwinii* was only detected in one soil sample (from Paplaka, in a sample, where *G. quercinecans* was also detected).

Assessment of fungi

A total of 23 fungal taxa were identified from wood core samples collected from the stem bases of oaks with visual symptoms of AOD and visually healthy trees. Fourteen isolates were identified at the species level, and nine at the genus level (Supplement Table 1). A total of 14 taxa were isolated from individuals with visual AOD symptoms, and 14 taxa were isolated from healthy trees. Five taxa were identified in both healthy and infected oaks (*Ascocoryne cylichnium* (Tul.) Korf, *Clonostachys rosea* (Link) Schroers, Samuels, Seifert et W. Gams, *Mucor* spp., *Penicillium* spp. and *Trichoderma* spp.).

A total of nine fungal taxa were isolated from symptomatic oak trees from the site of bark cracks, including two weakly pathogenic taxa (*Fusarium* spp. and *Sporotrix* sp.). While these fungi may not directly cause death of the oak individuals, they can weaken tree defences, and increase susceptibility to infection by additional pathogens.

A survey of fungal fruiting bodies in the trial plots, both on oak individuals as well as on ground cover. A total of 63 fungal taxa were found in all sample plots. The most commonly found taxa were identified in all plots, *Hebeloma crustuliniforme* (Bull.) Quél. and *Mycena galericulata* (Scop.) Gray. Four of the detected fungal taxa are considered to be capable of degrading both living and dead wood (*Armillaria cepistipes* Velen., *Daedalea quercina* (L.) Pers., *Grifola frondosa* (Dicks.) Gray and *Phellinus robustus* (P. Karst.) Bourdot et Galzin).

Assessment of soil pH, fine root vitality and mycorrhiza

The average soil pH in the Cīrava and Kazdanga plots was 4.0, 5.1 in the Šķēde 1 plot, and 4.3 in the Šķēde 2 plot. Soil pH was higher in the vicinity of symptomatic trees than from the vicinity of visually healthy trees (4.7 and 4.4, respectively), but the difference was not significant. The majority of fine roots were healthy in both symptomatic and healthy trees (61% and 54%, respectively, no significant difference). Fine root vitality between the sample

plots had no significant difference. All detected mycorrhiza types were present in fine roots of both symptomatic and healthy trees.

Correlation of Gibbsiella quercinecans and Brenneria goodwinii detection with meteorological factors

From a total of 111 exudate samples tested in the National Phytosanitary Laboratory between 2017 and 2021, 36 samples were positive for one or both AOD related bacteria. Three samples were positive only for *G. quercinecans*, 22 samples only for *B. goodwinii*, but 11 samples were positive for both bacteria.

The effect of daily precipitation (mm) on the presence of one or both bacteria *G. quercinecans* and *B. goodwinii* in samples is shown in Figure 8.

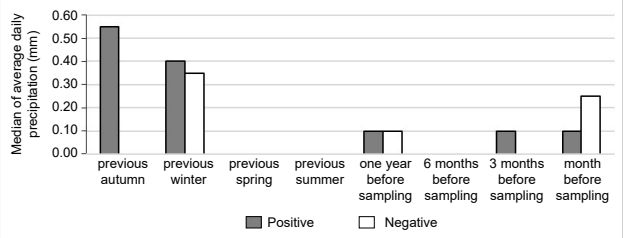

Figure 8. Comparison of median average daily precipitation (mm) records in the positive and negative sample groups (time points on x-axis relative to sample collection time)

There was a statistically significant difference (p (0.00) < 0.05) between the median of previous autumn daily precipitation (mm) in the positive (0.55 mm) and negative (0.00 mm) sample groups. Also, a moderate positive correlation ($\tau = 0.42$) was identified between the positive samples and the median previous autumn daily precipitation (mm).

A statistically significance difference (p (0.04) < 0.05) between the previous spring's median daily average temperature, °C, was detected in the positive and negative sample groups. Also, there was a weak positive correlation ($\tau = 0.20$) between the positive samples and the previous spring median daily average temperature, °C (Figure 9).

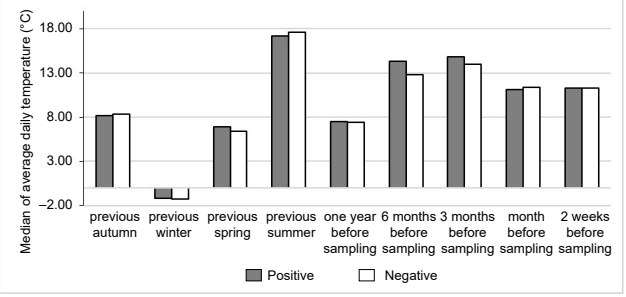

Figure 9. Comparison of median average daily temperature (°C) records in the positive and negative sample groups (time points on x-axis relative to sample collection time)

Statistical significance difference between the positive and negative sample groups was not detected according to median average daily relative humidity (%). Also, no correlation between the median average daily relative humidity (%) and the detection of one or both bacteria in exudate samples was identified (Figure 10).

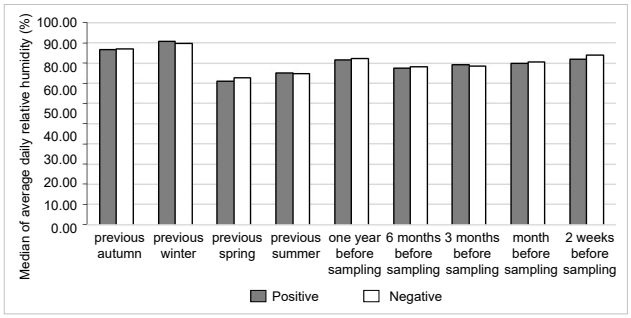

Figure 10. Comparison of median average daily relative humidity, %, records in the positive and negative sample groups (time points on *x*-axis relative to sample collection time)

Discussion

This study surveyed the occurrence of AOD symptoms (dark, sticky exudate from bark cracks) in oak stands throughout Latvia, as well as a more detailed monitoring of the development of symptoms in four trial plots. The presence of two bacterial species, *Gibbsiella quercinecans* and *Brenneria goodwinii*, that are associated with AOD (Brady et al. 2022) was assessed. Other bacteria, such as *Rahnella victoriana* and *Lonsdalea britannica*, are also associated with AOD, but *G. quercinecans* and *B. goodwinii* are reported to play key roles in the development of AOD in oaks (Crampton et al. 2020). These bacteria were detected in bark exudates collected throughout Latvia, both in oak stands and in individual trees. The bacteria were also detected in oak wood and soil samples collected from or near both symptomatic and healthy trees, as well as in oak stands where no individuals with AOD symptoms have been observed. This is in agreement with previous studies indicating that these bacteria are present in the oak microbiome, and that their presence alone is not sufficient to induce AOD symptoms (Gathercole et al. 2021). Although present in Latvia as a species, individuals or larval galleries of the beetle *Agrilus biguttatus* were not detected either in the monitoring plots or during the surveys of oak stands throughout Latvia. Confirmed records of this species were noted as recently as 2023 in the Latvian nature observation web site (nekovārnis 6.10.2023). *A. biguttatus* is considered to be rare species in Latvia and is included in the B list of Special Biotope Species (Zemkopības ministrija 2001). Previous studies in the United Kingdom have indicated a high correlation between AOD symptoms and the presence of *A. biguttatus* (Brown et al. 2015, 2017). In the course of this study, a potential vector for the spread of the bac-

teria in Latvia was not identified. This may be related to the relatively few exudate samples positively identified as containing one or both bacteria and also to the reduction in the presence and severity of visual AOD symptoms in the monitoring plots after 2019, possibly due to low precipitation during the sampling season and in preceding periods.

The presence of bark exudate and the ability to detect bacteria in collected samples may be dependent on weather conditions. The bacteria *G. quercinecans* and *B. goodwinii* are not always present in exudate samples and their incidence can vary over a single season in exudate collected from a single oak tree. Both bacteria were detected in soil samples, including soil samples collected from the oak stands where no visual symptoms of AOD have been detected. Bacteria are most commonly found in soil samples collected directly by oak individuals. *G. quercinecans* was detected in soil samples more frequently than *B. goodwinii*, which is consistent with reports that *G. quercinecans* is more likely to survive in soil and rainwater compared to *B. goodwinii* (Pettifor et al. 2020). This suggests that the bacteria are present in oak individuals and stands, as reported previously (Gathercole et al. 2021) and their presence does not always result in AOD symptoms (dark, sticky exudate from bark cracks). The appearance of AOD symptoms may be a result of more complex interactions between biotic and abiotic factors.

In this study, environmental factor analysis indicated that higher precipitation in the previous autumn is related to a higher detection possibility of *G. quercinecans* and *B. goodwinii* in exudate samples collected in the following year. Previous autumn precipitation in areas where negative exudate samples were collected was low, with no precipitation recorded on most days. It is known that exudate on the bark can dry out (Denman and Webber 2009), and dry weather conditions are not suited for bacterial survival (Grinberg et al. 2019). In contrast, higher previous autumn median average precipitation suggests that growing conditions were more suitable for both oak trees (Eaton et al. 2016) and bacteria (Grinberg et al. 2019). However, high precipitation can also make the detection of bacteria in exudate more difficult, as the exudate can be washed from the bark in heavy rain, making it difficult to sample and obtain reliable results (Denman and Webber 2009), this could be also one reason why we observed only a moderate positive correlation ($\tau = 0.42$) between positive samples and the median daily precipitation (mm) from the previous autumn. This is in contrast with previous studies of the correlation of environmental factors with AOD in the UK, where low rainfall was associated with affected sites (Brown et al. 2018). The majority of exudate samples in the countrywide survey (111 samples in total) were collected in autumn (59% of all collected samples). Of the exudate samples collected in autumn 42% were positive for one or both bacteria. In comparison, 36% of samples collected in spring were positive (4 of 11 samples), and only 14% of samples collected in summer were positive (5 of 35 samples). This may indicate that detection of these bacteria was lower in summer, but the sample sizes are too small to identify any trends with a high degree of confidence.

In the countrywide survey of oak stands, of 1,329 forest stands surveyed, only 111 exudate samples were collected, and of these only 36 were positive for one or both bacteria. Less than a third of the collected exudate samples tested positive, indicating that *G. quercinecans* and *B. goodwinii* were not detected in the majority of bark exudate samples. One possible explanation for the low detection incidence of these bacteria in exudate samples could be related with the age of the exudates at the time of sample collection. While the exudate samples were still moist when collected, due to the surveying method, the exudate may have been present for a longer period before collection, and the bacteria were no longer present in the exudate at the time of collection. This was supported by results from the analysis of exudate from oak trees in the experimental trial plots, where exudate was collected approximately every two weeks but a large number of individuals with fresh exudate and no rapid development of AOD symptoms were observed. The analysis by qPCR indicated that only three samples were positive for one or both bacteria indicating that the freshness of the exudate may influence the detection of the bacteria. Other bacteria have also been associated with AOD symptomatic oak trees, e.g. *R. victoriana* (Brady et al. 2017). During this study, samples were not tested for additional bacterial species associated with AOD, but such testing may yield more insights into bacterial species found in the exudates. Sampling of exudate and necrotic tissue in different seasons and years would provide more information about bacterial detection and composition differences between samples. High-throughput sequencing (HTS) data obtained from exudate, wood, and soil samples would provide more detailed information about bacterial communities associated with oak trees in Latvia, as well as with individuals with AOD symptoms. This information could provide more information about the bacterial life cycle and knowledge of the most appropriate sampling times, which is very important to obtain reliable laboratory testing results.

The composition of fungal taxa in the wood of symptomatic and visually healthy oak trees does not differ significantly, and no difference in soil pH, fine root vitality, and mycorrhization was found. The two most commonly found taxa, identified in all plots, *Hebeloma crustuliniforme* (Bull.) Quél. and *Mycena galericulata* (Scop.) Gray, are both very common species in Latvia, of which the first is a mycorrhiza-forming species, while the second is a saprotrophic species. Nine rare or very rare fungal taxa were found in the sample plots, three of which, *Grifola frondosa* (Dicks.) Gray, *Hymenopellis radicata* (Relhan) R.H. Petersen and *Otidea onotica* (Pers.) Fuckel, are specially protected species in Latvia. The obtained results indicate that the AOD monitoring plots have a diverse composition of fungal species, and that rare and protected fungal species

were identified in the monitoring plots, indicating that future management plans should take this into account.

The results of this initial survey and short-term monitoring of AOD in Latvia indicate that the disease and symptoms of AOD may be cyclical (over several years). Oak trees with symptoms of AOD do not always die, and oak trees can recover, even in individuals with severe symptoms (multiple bark lesions with exudate). The presence of *A. biguttatus* in AOD symptomatic oak trees was not detected in this study, and other potential bacterial or other pathogen vectors were not identified in this study. *G. quercinecans* and *B. goodwinii* were detected in oak stands that did not contain any oak trees with visual AOD symptoms, indicating that AOD may be a result of the complex interaction of several biotic and abiotic stress factors, which may then facilitate the development of AOD symptoms. However, these two bacteria were not detected in the majority of bark exudate samples collected during the countrywide survey, suggesting that AOD-like symptoms can develop in the absence of these bacteria (and also the absence of the beetle *A. biguttatus*), or that the bacteria were not present in the exudate at the time of sampling. Continued monitoring of oak stands throughout Latvia is required to determine the periodicity and spread of AOD symptoms in Latvian forests. Currently, the health condition of oak stands in Latvia concerning AOD is not critical, but further research and monitoring are required.

Conclusions

Visual AOD symptoms were first detected on oak (*Quercus robur* L.) trees in Latvia in 2017, and the presence of the bacteria *Gibbsiella quercinecans* and *Brenneria goodwinii* in the bark exudates were also confirmed. In a countrywide survey of 1,329 oak stands over four years, a total of only 111 bark exudate samples were collected, of which 36 were positive for the presence of one or both of the bacteria *G. quercinecans* and *B. goodwinii*. Larval galleries or other signs of the beetle *Agrilus biguttatus* were not found in association with AOD symptoms on the surveyed oak trees in Latvia. Observations (2018–2021) from the trial plots established in the oak stands, where AOD symptoms and the presence of the bacteria *G. quercinecans* and *B. goodwinii* were confirmed, indicated that visual AOD symptoms peaked in 2019, but many symptomatic oak trees recovered over the study period. Presence of the bacteria *G. quercinecans* and *B. goodwinii* was not detected in all exudate samples collected approximately every two weeks from trees previously having bark lesions with exudate in which the presence of these bacteria was confirmed. Assessment of fungal species in both symptomatic and healthy tress did not identify significant differences in species composition, as well as significant differences in soil pH, fine root vitality, and mycorrhization. In the absence of widespread incidence of AOD symptoms on oak trees in Latvia, the low prevalence of the bacteria *G. quercinecans* and *B. goodwinii* in bark exudates, and the absence of the beetle *A. biguttatus* or other possible insect vectors, standard phytosanitary precautions should be observed. This study found a significant correlation between AOD symptoms and tree diameter in the trial plots, suggesting that young oak individuals may be less susceptible to AOD in Latvia, but additional studies are required to confirm this. Continued monitoring for AOD symptoms, focussing on mature oak trees, is required to identify future potential AOD outbreaks in Latvia.

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Supplement

Table S1. Fungal taxa in AOD sample plots identified in samples collected from the root collar of healthy and symptomatic trees, bark lesions, and fruiting bodies present in the sample plots

Fungal taxa	Healthy trees	Symptomatic trees	Lesions	Sample plot
Amanita muscaria (L.) Lam.				x
Amanita virosa Bertill.				x
Annulohypoxylon multiforme (Fr.) Y.M. Ju, J.D. Rogers et H.M. Hsieh	x			
Armillaria cepistipes Velen.		x		x
Ascocoryne cylichnium (Tul.) Korf	x	x		
Cladosporium sp.		x		
Clonostachys rosea (Link) Schroers, Samuels, Seifert et W. Gams	x	x		
Collybia asema (Fr.) Gillet				x
Collybia sp.				x
Coniochaeta hoffmannii (J.F.H. Beyma) Z.U. Khan, Gené et Guarro		x		
Coniophora puteana (Schumach.) P. Karst.		x		
Cordycipitaceae sp.	x			
Cortinarius caerulescens (Schaeff.) Fr.				x
Cortinarius multiformis Fr.				x
Cortinarius sp.				x
Craterellus cornucopioides (L.) Pers.				x
Crepidotus sp.				x
Cyathus striatus (Huds.) Willd.				x
Daedalea quercina (L.) Pers.				x
Dothiorella sp.		x		
Fomes fomentarius (L.) Fr.				x
Fomitopsis pinicola (Sw.) P. Karst.				x
Fusarium avenaceum (Fr.) Sacc.			x	
Fusarium sp.			x	
Ganoderma applanatum (Pers.) Pat.				x
Grifola frondosa (Dicks.) Gray				x
Gymnopus acervatus (Fr.) Murrill				x
Gymnopus dryophilus (Bull.) Murrill				x
Gymnopus fusipes (Bull.) Gray				x
Hebeloma crustuliniforme (Bull.) Quél.				x
Hebeloma pusillum J.E. Lange				x
Hyaloscyphaceae sp.		x		
Hymenochaete rubiginosa (J.F. Gmel.) Lév.				x
Hymenopellis radicata (Relhan) R.H. Petersen				x
Hypholoma fasciculare (Huds.) P. Kumm.				x
Hypoxylon rubiginosum (Pers.) Fr.	x			
Inocybe geophylla (Bull.) P. Kumm.				x
Laccaria amethystea (Bull.) Murrill				x
Laccaria laccata (Scop.) Cooke				x
Lactarius necator (Bull.) Pers.				x
Lactarius quietus (Fr.) Fr.				x
Lactarius rufus (Scop.) Fr.				x
Leccinum scabrum (Bull.) Gray				x
Lepiota cristata (Bolton) P. Kumm.				x
Lepista flaccida (Sowerby) Pat.				x
Lepista nuda (Bull.) Cooke				x
Lycoperdon perlatum Pers.				x
Lycoperdon pratense Pers.				x
Lycoperdon pyriforme Schaeff.				x
Marasmiellus confluens (Pers.) J.S. Oliveira				x
Marasmiellus ramealis (Bull.) Singer				x
Mariannaea elegans var. *elegans* (Corda) Samson	x			
Megacollybia platyphylla (Pers.) Kotl. et Pouzar				x
Metapochonia bulbillosa (W. Gams et Malla) Kepler, S.A. Rehner et Humber		x		
Mucor spp.	x	x	x	
Mycena galericulata (Scop.) Gray				x
Mycena inclinata (Fr.) Quél.				x
Mycena polygramma (Bull.) Gray		x		x
Mycena pura (Pers.) P. Kumm.				x
Mycena rosea Gramberg				x
Mycena sp.				x
Nemania serpens (Pers.) Gray	x			

Table S1. (continued)

Fungal taxa	Healthy trees	Symptomatic trees	Lesions	Sample plot
Obolarina dryophila (Tul. et C. Tul.) Pouzar	x			
Otidea onotica (Pers.) Fuckel				x
Paxillus involutus (Batsch) Fr.				x
Penicillium spp. Link	x	x	x	
Phallus impudicus L.				x
Phellinus robustus (P. Karst.) Bourdot et Galzin				x
Pholiota squarrosa (Vahl) P. Kumm.				x
Pluteus phlebophorus (Ditmar) P. Kumm.				x
Polyporus badius (Pers.) Schwein.				x
Psathyrella candolleana (Fr.) Maire				x
Psathyrella piluliformis (Bull.) P.D. Orton				x
Psilocybe sp.				x
Querciphoma minuta (J.C. Carter) Crous et P.M. Kirk			x	
Ramaria stricta (Pers.) Quél.				x
Rickenella fibula (Bull.) Raithelh.				x
Russula xerampelina (Schaeff.) Fr.				x
Simplicillium sp.	x			
Sporothrix sp.	x		x	
Tremella foliacea Pers.				x
Trichoderma spp.	x	x	x	x
Trichoderma viride Pers.	x		x	
Tricholoma sp.				x
Tricholoma sulphureum (Bull.) P. Kumm.				x
Truncatella angustata (Pers.) S. Hughes		x		
Unidentified yeast			x	
Total	14	14	9	63