

Article

Pine Stands as Bioindicators: Justification for Air Toxicity Monitoring in an Industrial Metropolis

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Abstract: Five permanent sample plots (SPs; 200–250 trees per plot) were established in middle-aged high-grade suburban pine stands near the industrial city of Krasnoyarsk, Siberia, Russia. Needle damage, inventory parameters of the stands, and the defense response of the stem phloem were evaluated annually for the years 2002–2019 and attributed to acute or chronic toxic exposures (creeping fire or industrial pollutants, respectively). The results form a basis for using trees as bioindicators. A newly elaborated stem lesion test was formed from a hypothesis on the upward sugar transport for the regeneration of an injured crown, based on Eschrich’s model of bidirectional sugar transport in the phloem. The formation of a phloem lesion was induced by inoculation of the stem with a mycelial extract of the ophiostomatoid fungus *Ceratocystis laricicola*. The lesion length and its shift relative to the inoculation hole were measured. An increase in the length of needles at early stages of stand weakening by pollutants was found to correspond to the hormesis model (Selye’s adaptation syndrome). A possibility of assessing the chronology of pollutant toxicity and the duration of the recovery period after creeping fire was shown.

Keywords: environmental monitoring; pollutants; toxicity; creeping fire; high-grade pine stands; bioindicators

1. Introduction

The diagnosis of early-stage ecosystem damage is important task from the perspective of preventive measures against toxic impacts. The term ‘toxicity’ is defined as an ability of substances to suppress the physiological functions of an organism. In plants, toxins may cause necrosis and death [1].

Air toxicity is assessed by chemical and biological tests. A chemical assay alone is not sufficient in toxicity evaluation for complex organisms due to: (1) non-additivity of pollutant effects and (2) the possible presence of unknown toxic compounds. Moreover, chemical analysis of air pollutants does not take into consideration previous toxic exposures and the accumulation of toxicants in soils and water. Biological assays are free from these disadvantages. The characteristics of test organisms integrate the toxic effects of different factors over prolonged periods. There exist two biological approaches to assess toxicity in natural environments; these are based on either bioassays or bioindicators. The bioassay procedure is carried out under laboratory conditions using living objects—plants, animals, or microorganisms. Such biological micro-objects as enzymes or fluorescent proteins are used as

well [2]. Effects of water, air, and soil samples on the bioassay systems are monitored over the course of the bioassay procedure. The advantages of the bioassays are that they use proper sizes of bio-objects and there is the possibility of applying laboratory equipment.

Bioindication involves observation of biological objects under natural conditions [3]. Along with other bio-objects, herbaceous and woody plants can be used as bioindicators. Changes in morphological, histological, and biochemical characteristics, as well as the accumulation of contaminating elements in tissues, are studied [4,5].

The impact of air pollutants on forests has been intensively discussed [3,4,6,7]. Unfortunately, the assessment of inventory and physiological parameters of trees and stands related to air contamination, as a rule, has not attracted the attention of researchers.

The use of tree stands as bioindicators allows for long-term studies in permanent sample plots (SPs) to be performed. This allows us to trace the long-term dynamics of the vigor state of individual trees and the health status of forest stands, as well as the chronology of air toxicity.

We chose suburban forests near the city of Krasnoyarsk, the Siberian megalopolis of Russia. Two middle-aged pine stands were the focus of the investigation. Standard inventory parameters of these pine stands (average diameter, average height, stock, depth, degree of density, etc.) were annually evaluated in permanent SPs for the period 2002–2019. These parameters were compared to the results of the stem lesion test applied simultaneously in the pine stands.

The current study elaborates a method for assessing environmental toxicity using pine stands as bioindicators. Our previous investigations [8–10] show the promise of a stem lesion test for assessing the impact of environmental factors on the vigor state of conifer trees. The stem lesion test applies characteristics of the stem phloem defense response after inoculation with the mycelial extract of ophiostomatoid fungi, as well as live mycelium. As for pine trees, the use of the mycelial extract of the ophiostomatoid fungus *Ceratocystis laricicola* is promising [8,9].

2. Materials and Methods

2.1. Establishment for the Permanent Sample Plots in the Pine Stands

Studies were performed in the suburban middle-aged high-density pine stands of Krasnoyarsk, a large industrial center in Siberia. The world's largest Söderberg aluminum smelter (which produces more than 1 million tons of aluminum per year) is located in Krasnoyarsk. Söderberg smelters emit polycyclic aromatic hydrocarbons (including benzo[a]pyrene) and fluorine compounds [11,12]. In Krasnoyarsk, this has caused high pollution of the urban and suburban ecosystems with toxic cancerogenic compounds [12]. Benzo[a]pyrene concentrations in the air are sometimes ten times higher than the permissible level. Krasnoyarsk often takes the final position in the Ranking of World air quality index (AQI), and has the highest level of air pollution [13].

Two pine stands, 'A' and 'B', were chosen. Five permanent sample plots (SPs) were established in the pine stands in accordance with the Directions for Forest Management [14] and Forest Regulations [15]: SPs 1–4 in 2002, and SP 5 in 2005 [16]. An individual number was assigned to each tree in the SPs (Figure 1).

About 200–250 trees grow in each SP. The predominant species is Scotch pine (*Pinus sylvestris* L.) (150–200 specimens in each SP).



Figure 1. Numbered trees in permanent sample plot 2 in a middle-aged pine stand in 2019.

All SPs are situated approximately at the same distance from the city margins (about 7 km). SPs 1, 2, and 5 were established in pine stand 'A' ($56^{\circ}02'25''$ N, $93^{\circ}08'54''$ E, 160 m above sea level), while SPs 3–4 were in pine forest 'B' ($56^{\circ}01'55''$ N, $92^{\circ}40'52''$ E, 270 m above sea level). The pine stand 'A' is located in the direction of dominant winds from the industrial metropolis. In contrast to 'A', the 'B' pine stand is located at the opposite side of the metropolis. A higher degree of anthropogenic pollution in forest area 'A', as compared to 'B', was reported in 2002 when the SPs were established [16]. The result was confirmed by the data of the ground and space sensing of the snow cover. The wind rose remained unchanged in the city of Krasnoyarsk over the years 2002–2019. Southwest winds prevailed.

Pine stands 'A' and 'B' do not differ in forest conditions (forest-steppe zone, Central Siberia) and forest type (grass-green moss pine forest) [17]. Soils in pine stand 'A' and 'B' were identified as grey forest soils.

While selecting plots for the SPs, special attention was paid to the similarity of tree stands in terms of age, composition, stock, and other inventory parameters [16] (Table 1).

Table 1. Inventory parameters of forest stands in permanent sample plots (SPs) in the establishment year and in 2019.

SP No.	Year	Stand Composition	Degree of Density ¹	Principal Forest Element	Mean			Stock, m ³ /ha		Depth ³ , ind/ha	
					Age, Years	Height, m	Dia-Meter, cm ²	Green Timber	Dead-Standing Trees	Green Timber	Dead-Standing Trees
1	2002	10P ^{II} +P ^I ⁴	1.4	P ^{II}	57	18.1	18.5	396	6	1671	56
1	2019	10P ^{II} +P ^I	1.6	»	74	21.7	22.6	550	17	1360	119
2	2002	9P ^{II} +P ^I rB	1.5	P ^{II}	58	21.3	21.9	489	10	1286	36
2	2019	10P ^{II} +P ^I	1.6	»	75	25.4	26.3	658	16	1043	86
3	2002	9P ^I B r.L	1.5	P	62	21.1	20.4	472	8	1430	81
3	2019	9P ^I B r.L	1.6	»	80	25.2	25.2	616	29	1067	163
4	2002	9P ^I L+B	1.5	P	62	20.9	19.6	503	9	1664	172
4	2019	9P ^I L+B	1.6	»	81	25.5	25.5	628	32	1055	227
5	2005	9P ^{III} P ^I	1.5	P ^{II}	59	16.3	16.4	339	3	1980	41
5	2019	9P ^{III} P ^I	1.6	»	73	18.7	19.0	433	10	1682	88

¹ Degree of density, the ratio of the actual basal area at 1.3 m to the standard one (the latter was taken from the tables of the basal area and the volume of normal growing stock for pine [18]). ² Trunk inventory diameter at a height of 1.3 m. ³ The depth units, ind/ha, correspond to the number of individual trees per hectare. ⁴ P^I and P^{II}, pines of the first (parent) and second (daughter) generations, respectively; B, birch; L, larch; 10, 9, 1, +, r, proportions of the species (forest element) in the stand determined from the green timber stock: 100, 90, 10, 2–5, and below 2%, respectively.

For all SPs, the inventory parameters of pine stands had been evaluated annually. In the establishment year, a trunk inventory diameter of each tree at a height of 1.3 m was measured. The height and age (by annual layers of stem cores) of the selected trees were also measured. In August, a vigor state category of each tree was defined using the 6-point scale of Sanitary Regulations in the Forests of the Russian Federation [19]. According to the 6-point scale, category I includes trees without signs of weakening, category II includes weakened trees, category III includes severely weakened trees, category IV includes suppressed trees, category V includes standing trees which died in the current year, and category VI includes standing trees which died in the previous years. The vigor rank of the tree stand was a weighted average for trunk volumes.

Modeling and annual data updates had been performed using a database developed in the MS Access application [16]. The database accumulated annual observations and provided equations describing the relationship of different parameters: diameter and annual increment in diameter, vigor state category and annual increment in height and diameter, and height and age, etc.

A high density of pine stands was noted, at 1.4–1.6 times the standard density. The high density of pine forests, exceeding the maximum standard values of the Tretyakov's tables [18], has also been noted by other authors [20,21].

High-grade pine stands are known to consist of even-aged populations of trees. These pine stands are characterized by a differentiation of trees: their division into classes depends on the size. Large trees are generally more resistant than small ones. The development and growth of a stand imply the dying out of weakened individuals. The magnitude of tree fall had been recorded in the SPs annually. The low light inside the stand of high density causes branches to fall off the trunk (Figure 1), forming small-tapering trunks with high crowns.

The length of two-year needles in the upper parts of crowns is one of the test parameters in pine forests. Branches of 8–9 trees (fresh litter) were collected in SPs from the surface of the snow cover during the period of strong winds in November 2007 and 2016.

2.2. Acute and Chronic Impacts on Pine Stands

The height of soot deposits on trunks up to 2 m was registered after a strong spring creeping fire in stand 'B' in 2004. Necrotization (discoloration) of 10–15% of the needles of all trees in SPs was registered in stand 'A' in 2012–2019.

2.3. Phytopathological Control of Needles in the SPs

For analysis, pine needles with discoloration were selected. To confirm or refute the presence of a fungal infection, the needles were cultivated in wet chambers for 21 days for sporulation and to identify the pathogen [22].

2.4. The Stem Phloem Defense Response (Stem Lesion Test)

We applied the stem lesion test developed in our laboratory [8–10]. This test is based on the well-known method of stem inoculation with live mycelium of an ophiostomatoid fungus and induction of conifer plant defense response [23–26].

The ophiostomatoid fungi are known to inhabit conductive tissues of conifer species. The fungi are vectored by xylophagous insects [23,25].

Our preliminary investigations show that the phloem defense response evolves after inoculation of mycelium extract as well as live mycelium [8–10,24]. A phloem lesion was formed. After 4 weeks, the lesion reached final dimensions, since the insulating periderm had already formed by that time, dividing necrotic and living tissues (Figure 2). A lesion length depends on a transport of fungal elicitors from the inoculation point. This allows us to suggest that a lesion asymmetry was caused by an activation of the sugar phloem transport to crowns or roots.



Figure 2. A lesion in the pine stem phloem (after removal of the dead bark) 4 weeks after 0.5-mg injection of an extract of *Ceratocystis laricicola* fungus mycelium into the inoculation hole.

Pine trees of vigor state categories 1–3 were annually selected randomly (20–25 trees in each SP) and inoculated. The extract from mycelium of the ophiostomatoid fungus *C. laricicola* (Redfern @ Minter) was used. The inoculation was carried out in the middle of July. In the bark of the trunk, a 7-mm diameter sapwood hole was cut, and 50 μ L of solution containing 0.5 mg fungal extract were injected into the hole that was closed by bark core. The optimal dosage of the fungal extract was determined by additional studies [16].

The size of the developing necrosis was measured 4 weeks after inoculation. The size of the upper part of the lesion (the distance from the upper margin of the lesion to the inoculation hole) was also measured. The vertical asymmetry of the lesion was calculated as a relation of the upper part length to

the total length. We assume that this allows for determining a direction of the dominant transport of assimilates in the stem phloem, downward or upward.

2.5. Preparation of the Fungal Extract

A pure culture of the *C. laricicola* fungus was grown on liquid beer wort by the ‘batch culture’ method at 24 °C. The 7-day mycelium of the fungus on wort agar was used as an inoculum for a liquid medium. After 10 days of cultivation in liquid medium, the fungal biomass was collected by filtration. The pre-frozen mycelial mass was used to prepare the fungal extract. The mycelium was homogenized using liquid nitrogen and ultrasound (15 kHz) with the addition of silica sand. After an exhaustive extraction with 70% ethanol, the supernatant was evaporated to dryness. The residue dissolved in water (5 mL) was dialyzed against 4 L of water for 2 days with a 4-fold change of water at 4–6 °C; [10].

3. Results and Discussion

3.1. Vigor Rank of Pine Stands, Needle Necrosis, and Fall (Death) of Trees

Annual monitoring in 2002–2019 showed a trend of vigor rank increase for pine stand ‘B’ (Figure 3), indicating a deterioration of its state. The rate of vigor rank increase for pine stand ‘B’ was maximal after 2004 and 2012. The latter was accompanied with needle necrosis visually recorded in the upper parts of crowns. Necrotization of 10–15% of needles of all trees in SPs was registered.

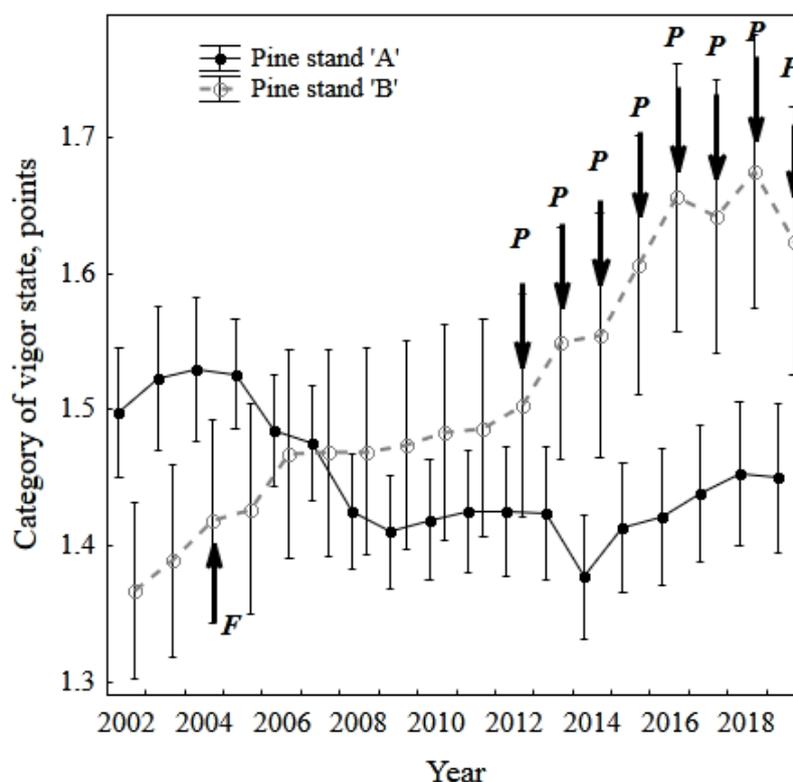


Figure 3. Category of vigor state of pine stands ‘A’ and ‘B’ in permanent sample plots over the years 2002–2019 (an increase in the score of the category corresponds to the deterioration of their condition). Acute or chronic impacts on the stands were caused by a thermal factor (*F*, a creeping fire) or chemical factor (*P*, pollutants), respectively. Average values for 300–400 trees and their standard errors are shown.

A spring creeping fire took place in 2004 in pine stand 'B' (marked with *F* in Figures 3–5 and 7). A creeping fire in pine stand 'A' was not registered, and pine stand 'A' did not demonstrate an increase in the vigor rank. Hence, an assumption can be made that the creeping fire in pine stand 'B' induced the increase in the vigor rank.

Additionally, the fall (death) of trees was observed in 2004 (Figures 4 and 5) in pine stand 'B', which can also be attributed to the creeping fire. The fall increase was significant as compared to pine stand 'A' ($p < 0.05$).

Therefore, our results show that an increase in the vigor rank and fall of trees are interrelated in the pine stands; the change in these parameters in 2004 can be attributed to the spring creeping fire. The change in these parameters in 2012–2019 and their relation with needle necrosis is a question of special interest.

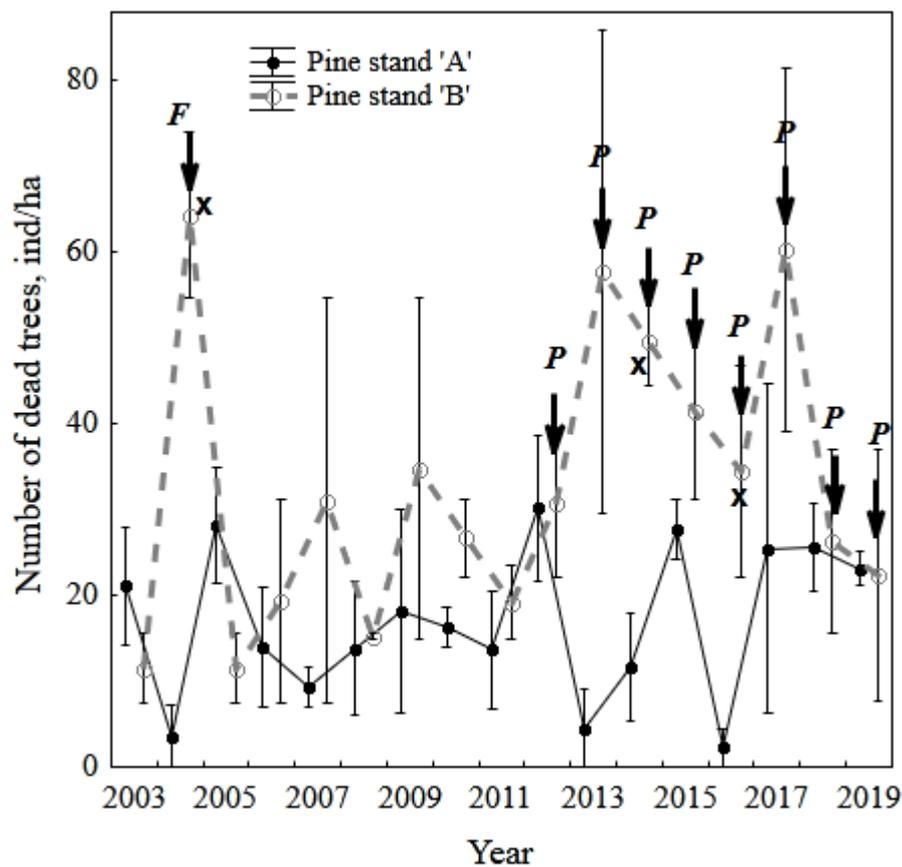


Figure 4. Number of trees which died in 2002–2019. Acute or chronic impacts on the stands were caused by the thermal factor (*F*, a creeping fire) or chemical factor (*P*, pollutants), respectively. “X” marks a significant difference between pine stands ‘A’ and ‘B’ ($p < 0.05$ by the *t*-test). Average values for 2–3 permanent sample plots and their standard errors are shown.

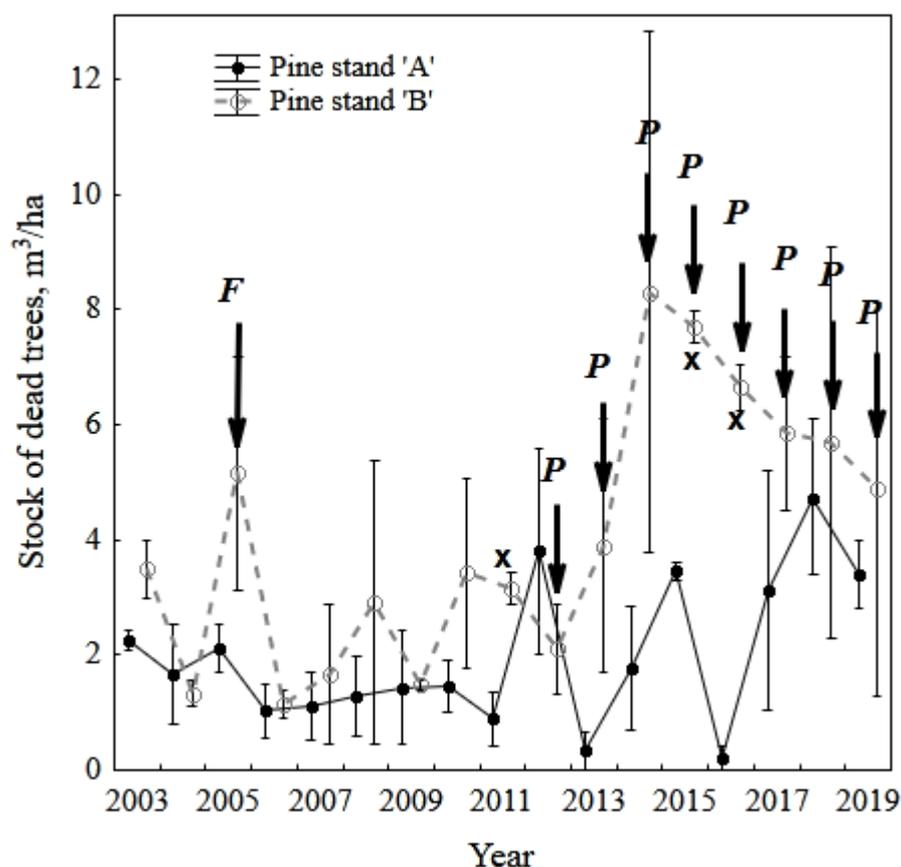


Figure 5. Stock of trees which died in 2002–2019. Acute or chronic impacts on the stands were caused by the thermal factor (*F*, a creeping fire) or chemical factor (*P*, pollutants), respectively. “X” marks a significant difference between pine stands ‘A’ and ‘B’ ($p < 0.05$ by the *t*-test). Average values for 2–3 permanent sample plots and their standard errors are shown.

We found that 10–15% of needles of each tree necrotized annually during 2012–2019 in pine stand ‘B’. Phytopathological analysis did not reveal any fungal infection in the necrotized needles. The damaged needles had an orange color (according to the scale of colors $\times 7$) [27]. As a rule, the needles died off entirely. In some cases, the needle tips were necrotized and their dead parts were separated from the live green ones with a narrow dark zone. In the book by Roll-Hansen and Roll-Hansen [28], similar changes in pine needles in the region of aluminum production were associated with damage by fluoride compounds. The authors explain the needle tip necrotization by the toxicant transmission to the tip of the needles as a result of transpiration process. Hence, the pattern of the needle damage in 2012–2019 can indicate the injury of the needles by air pollutants. It can be assumed that the massive needle necrosis of pine stand ‘B’ was caused by toxic airborne pollutants from the Krasnoyarsk Aluminum Smelter. The impact of air pollutants on the pine stands during 2012–2019 is marked with letter *P* in Figure 3, Figure 4, Figure 5 and Figure 7. These figures demonstrate that an increase in the vigor rank and fall of the trees were observed in this time-period as well.

A lower adaptability to toxicants of pine stand ‘B’ can be explained by a shorter period of exposure to the pollutants, in contrast to that for pine stand ‘A’.

The results indicate that severe damage to the needles by the chemical factor increases the fall. It should be noted that the strong creeping fire probably caused thermal damage of the needles in stand ‘B’ in 2004.

Thus, a comprehensive analysis of the parameters (needle necrotization, vigor rank of the pine stands, and fall of trees) reveals a connection of the condition of the trees with the external factors. The results form a basis for application of pine stands as bioindicators.

3.2. Needle Length and Selye's Adaptation Syndrome (Hormesis)

As noted before, both pine stands 'A' and 'B' are located apart from the industrial zone, 7 km from the city margins. Stand 'A' is located in the direction of prevailing winds from the city; stand 'B' is located on the opposite side of the metropolis. For this reason, stand 'B' served initially as a reference against stand 'A' exposed to pollutants.

Observations showed that in 2007, the length of needles in pine stand 'A' was larger than that in 'B' ($p > 0.05$) (Figure 6). This effect corresponds to Selye's adaptation syndrome, which suggests a mobilization of the protective properties of the organism at the initial stage of exposure to toxic factors. This stage is followed by weakening under chronic exposures [29,30].

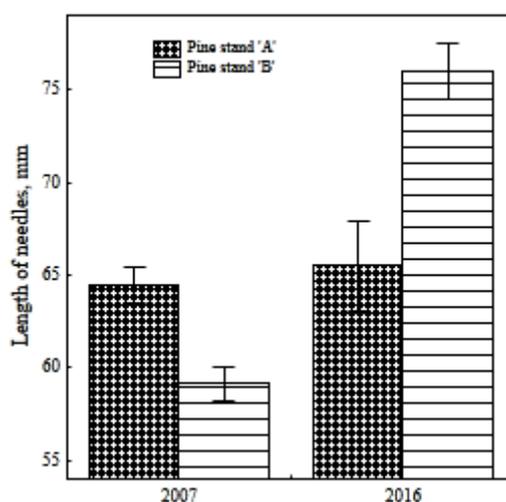


Figure 6. The length of 2-year pine needles sampled in 2007 and 2016 from upper parts of pine crowns. The average values for 120–200 needles sampled from 8–9 trees and their standard errors are presented. The length of the needles in pine stands 'A' and 'B' was significantly different in both 2007 and 2016 ($p < 0.05$).

Selye's syndrome is described by the 'hormesis' model, which suggests that low-dose effects induce activation of physiological functions; further chronic dose accumulation reduces the activating effect and evolves into an inhibitory (toxic) effect [31–35]. Probably, the increase in the size of needles in 2007 in pine stand 'A' allowed the trees to compensate the decrease in the productivity of photosynthesis when the needle surface was polluted and gas exchange was affected.

In contrast to 2007, in 2016 the length of needles in pine stand 'B' was larger than that in 'A' ($p > 0.05$) (Figure 6). As discussed previously in Section 3.1, pine stand 'B' is characterized by chronic needle burns owing to air pollution in 2016, along with the deterioration of condition and intensification of tree fall.

Thus, such a physiological parameter as the length of needles revealed an activation response corresponding to the initial stage of a toxic exposure [29,30]. It is evident that the length of needles differs from the other parameters (vigor rank of the pine stands and fall of trees), which showed an inhibitory (toxic) effect only.

3.3. The Stem Lesion Test (Inoculation of the Trunk with the Fungal Extract and Registration of Stem Phloem Necrosis)

Observations of pine stand 'A' showed that the necrosis was, as a rule, shifted downward from the inoculation hole, or exhibited such a tendency (Figure 7). Another pattern was observed for pine stand 'B': two 'peak (shifts)' of necrosis were noted upward of the trunk. The first 'peak' was registered in pine stand 'B' in 2004 after the strong spring creeping fire, with a height of soot deposits on trunks up to 2 m and thermal injuries of needles. The second 'peak' was recorded in 2013, within the period of

2012–2019, when mass necrotization of needles was registered in pine stand ‘B’ (see Section 3.1) as a result of toxic effects of airborne pollutants.

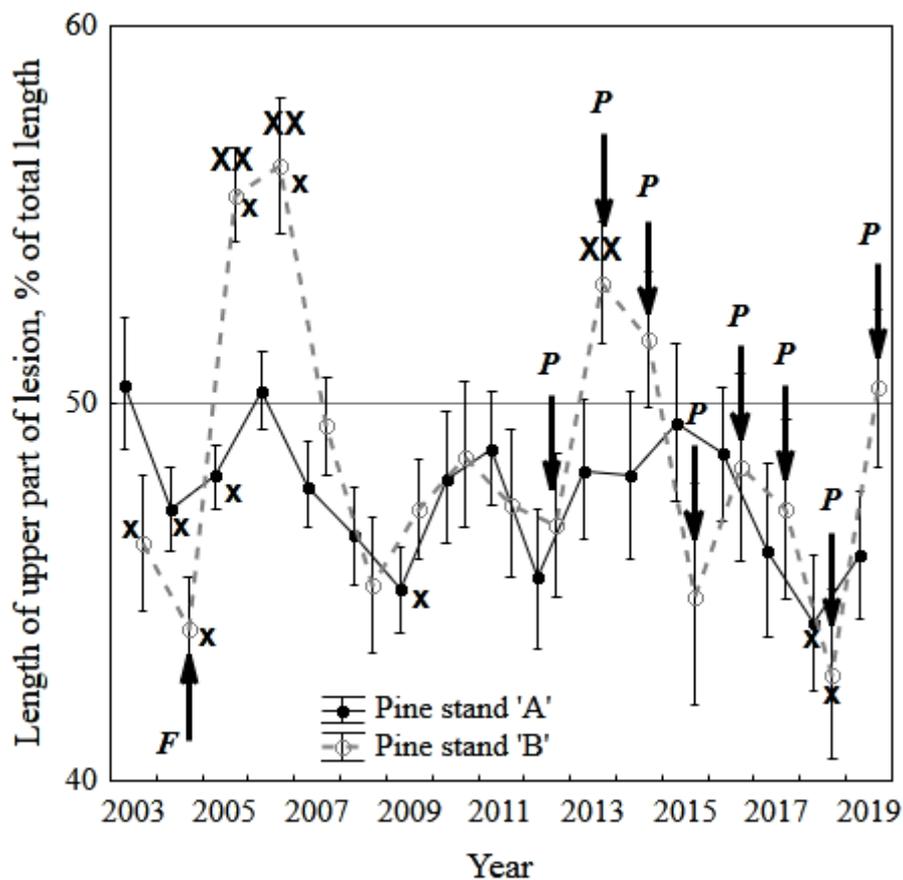


Figure 7. Shift of phloem necrosis relative to inoculation holes. Acute or chronic impacts were caused by the thermal factor (*F*, a creeping fire) or chemical factor (*P*, pollutants), respectively. “XX” marks a significant difference between pine stands ‘A’ and ‘B’ ($p < 0.05$ by the *t*-test), and “x” between the sizes of the upper and lower parts of necrosis according to the *t*-criterion ($p < 0.05$). Average values for 30–40 trees and their standard errors are shown.

As a rule, assimilates are known to be transported along the trunk phloem from the crown to the roots. However, in accordance to the concept of bi-directional transport, assimilates can also move in the opposite direction [36].

Based on the results obtained, we put forward a hypothesis explaining the relationship between the vertical displacement of necrosis of the stem phloem and damage of needles by environmental factors (chemical or thermal). The hypothesis is based on the assumption that fungal elicitors (which induced the phloem lesion) diffuse in the direction of assimilate transport to a greater degree. According to our hypothesis, any damage of crowns in stands changes the phloem transport from basipetal to acropetal. This hypothesis is consistent with the concept of bidirectional transport (translocation) of assimilates in the phloem [36].

In this case, the influx of assimilates into the crown from trunk tissues and roots occurs. The supply of needles with assimilates is improved, and the necrotic spot is shifted to the crown.

The results showed that in the case of chronic damage of crown needles by pollutants, the initial shift of necrosis upward the trunk take turns to a downward shift (Figure 7). Such a ‘return’ of necrosis to the ‘normal position’ corresponds to the second stage of Selye’s syndrome caused by tree depletion.

Creeping fire, in contrast to the chronic effects of pollutants, can be considered as an acute stress. In this regard, we associate the ‘return’ of necrosis to its normal position with a successful regeneration

of the needles, so that there is further need for the influx of assimilates into the crown from the storage tissues of trunk and root.

4. Conclusions

The present paper considers needle damage, inventory parameters, and a defense response of the stem phloem in pine stands as bioindicator parameters. These parameters were evaluated annually in 2002–2019 in middle-aged high-grade suburban pine stands near the industrial city of Krasnoyarsk, Siberia, Russia. Changes in the parameters were attributed to acute or chronic toxic exposures to creeping fire or industrial pollutants, respectively.

A steady weakening of suburban pine stand 'B' was registered over the period 2012–2019. This weakening was caused by chronic extensive needle damage with toxic pollutants. The source of these pollutants, apparently, is a powerful aluminum plant of the Krasnoyarsk city. The damage of needles caused an enhancement in fall of trees in pine stand 'B'; a similar increase in the fall was registered after the strong creeping fire in 2004. The damage of tree crowns by air pollutants was accompanied by an increase in the size of the surviving needles. Needle 'gigantism' was apparently related to the ability of the trees to compensate the damage of their photoassimilation apparatus. The needle increase can be discussed in terms of Selye's syndrome, i.e., an activation of the protective functions of the organism at the initial stage of a negative impact.

Our results show a prospect for early diagnosis of tree damage using a newly found effect induced by the ophiostomatoid fungus mycelium extract (i.e., stem lesion test). Inoculation of the extract induces the formation of phloem lesions. We found that toxic or thermal damage to the crown 'shifts up' the lesions relative to the inoculation hole. This effect can be explained in terms of the stem phloem defense response. The result corroborates our hypothesis on the upward transport of sugars along the trunk after thermal or toxic damage of needles, as well as Eschrich's concept of bi-directional transport of assimilates.

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