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Effects of wood distillate (pyroligneous acid) on sensitive bioindicators (lichen and moss)

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ABSTRACT

Wood distillate (pyroligneous acid) can be successfully applied in agriculture to increase crop quality and productivity with a lower risk for the environment respect to synthetic chemical herbicides, pesticides or fertilizers. However, the effects of wood distillate on the environment and biota are still under investigation, depending on biological attributes of potentially influenced organisms. The potential toxicological effects of wood distillate on sensitive non-target organisms, lichens and mosses, are studied for the first time. The physiological parameters (chlorophyll *a* fluorescence emission F_V/F_M and $PI_{(ABS)}$, chlorophyll content, spectral reflectance, antioxidant power, and dehydrogenase activity) and eventual bioaccumulation of selected elements (As, Ba, Cd, Cr, Cu, Fe, Ni, Pb, Zn) were investigated in the lichen *Xanthoria parietina* and the moss *Hypnum cupressiforme* after short-term treatments over a range of wood distillate solutions (1:300, 1:500, 1:700) to detect potential early stress responses. Overall, the lichen did not show changes after the treatments, while in the moss wood distillate caused only modest alterations in F_V/F_M and $PI_{(ABS)}$ and progressive increasing of antioxidant activity according to the dose supplied. The bioaccumulation of toxic elements was low and did not show any pattern of uptake with increasing concentrations of wood distillate.

1. Introduction

Globally, 140 billion metric tons of biomass waste are annually produced from agricultural and forestry practices (UNEP, 2009). Biomass residues are an increasing problem in many countries since their disposal, utilisation and management are not efficient (Tripathi et al., 2019) and their inappropriate processing, such as burning, may lead to serious environmental problems, e.g. degradation of soil biota, release of particulates, volatile and semi-volatile organic compounds, ash, sulphate aerosols and gases in the atmosphere, contributing to climate change, species extinction and health issues (Balat, 2006; Grewal et al., 2018). Strategic international actions to cope with this issue are promoted worldwide, as the United Nations set up waste reduction and disposal by environmental friendly practices as one of the main Sustainable Development Goals (United Nations, 2015). Therefore, sustainable technologies to minimize waste burning and convert plant biomass to useful bioproducts are widely encouraged (Grewal et al., 2018). For example, the use of renewable energy (including biofuels) in the European Union has doubled since 2005, reaching 18% of gross final energy use in 2018 (EEA, 2019). For bioenergy and biofuel production, the use of wood waste or industrial wood processing can be successfully implemented by means of modern low cost processes (Bhaskar et al., 2011). Biomass can be converted into gaseous, liquid and solid fuels by various processes starting from pyrolysis, a controlled-environment thermal decomposition in the absence of oxygen, followed by gasification and carbonization. There are several primary and secondary products of commercial value formed during this process, such as biochar, syngas, vegetable tar, and pyroligneous acid (also named as wood vinegar, bio-oil, wood distillate etc.), which are alternative to traditional fuels and inorganic chemicals (Bridgwater, 2003; Grewal et al., 2018; Lewandowski and Milchert, 2011).

Pyroligneous acid can be successfully applied in various agricultural practices to increase crop productivity and harvest quality with low environmental risk by replacing synthetic chemical herbicides or fertilizers (Grewal et al., 2018). Its complex chemical composition, mainly high content of acetic acid, phenols and esters, stimulates antioxidant (Loo et al., 2008; Ma et al., 2013; Wei et al., 2010), antimicrobial (Matsushita et al., 2005; Mmojieje and Hornung, 2015) and growth promotion properties of plants (Masum et al., 2013; Mungkunkamchao et al., 2013; Travero and Mihara, 2016) and

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thus impacts their growth, development and health. Pyroligneous acid produced under different conditions (residence time, heating rate, temperature, particle size, biomass feedstock) may slightly differ in its chemical composition, making also differences in characteristics and properties (Lee et al., 2010; Mathew and Zakaria, 2015; Theapparat et al., 2018).

In this study, we focus on wood distillate produced according to natural processes: it is considered as a biodegradable product, with no residues, featuring constant and safe characteristics for the environment (BioDea, 2020). It is extracted along temperature gradients using only the physiological water present in the sapwood; then it is passed through natural filters for the removal of any residue and left to settle for at least three months in order to obtain an amber-coloured distillate. According the producer, it contains more than 300 synergistically active organic substances (especially acetic acid, polyphenols and tannins) which favour the development of endogenous and exogenous plant defence mechanisms against biotic and abiotic stress, strengthen roots and stems, reduce evapotranspiration and improve the assimilation of microelements. It is mainly suggested to be employed in agriculture as revitalizer for plant development and soil quality (BioDea, 2020).

According to international regulations (EC, 2003), the ecotoxicological effects and ecological risk of such products on the environment should be assessed. Several studies investigated the impact of pyroligneous acid on non-target organisms, such as soil organisms (e.g. nematodes, enchytraeids, plants, microbes; Hagner et al., 2010a) and aquatic ones (e.g. crustacea, oligochaeta, mollusca, vascular plants, fishes, algae, bacteria; Hagner et al., 2010b). Nevertheless, to the best of our knowledge, side effects on mosses and lichens have not been studied, yet, despite they represent substantial part of biodiversity and possess unique traits that are directly related to ecosystem processes (Deane-Coe and Stanton, 2017). Mosses and lichens are well-known for being very sensitive to airborne pollutants since they take up nutrients and contaminants all over their surfaces and lack specific protection mechanisms, making them very sensitive and useful bioindicators of environmental contamination (Nash and Egan, 1988). For these reasons, lichens and mosses are widely used in biomonitoring studies of atmospheric pollution (Loppi and Bonini, 2000; Nash and Wirth, 1988).

In this study we evaluated possible negative effects of wood distillate on lichens and mosses. To this purpose, selected physiological parameters (photosynthetic efficiency, chlorophyll content, spectral reflectance, antioxidant power, and dehydrogenase activity) and the elemental content have been investigated in the moss *Hypnum cupressiforme* Hedw. and the lichen *Xanthoria parietina* (L.) Th.Fr., treated over a range of wood distillate concentrations. Our working hypothesis was that wood distillate does not significantly alter the vitality of the selected model bioindicators after short-term exposure.

2. Methods

2.1. Sample collection

The epiphytic moss *Hypnum cupressiforme* and the lichen *Xanthoria parietina* were selected owing to their wide distribution in Italy and being commonly used in biomonitoring studies, both in laboratory conditions and in the field (Bargagli et al., 2002; Loppi et al., 2006; Paoli et al., 2013, 2014a, Paoli et al., 2014, 2014c; Vannini et al., 2018a). Moss and lichen samples (ca. 20 tree branches with at least 10 independent individuals) were collected at the end of September 2019 (cumulative precipitation in 2019 was 1028 mm; source: database SIR, Regione Toscana, http://www.sir.toscana.it) in an area of central Italy (the province of Siena, Tuscany) far removed from any local source of air pollution (N43.217732° E11.376352° and N43.182552° E11.366710°). The samples were collected from the trunks and branches of *Quercus pubescens* trees which have a sub-acid bark pH of 5.2. The chemical content of the material growing in that area is known to reflect background values of unpolluted environments (Loppi and Paoli, 2015). After collection, lichens and mosses were left at constant conditions of 16 °C, RH = 50% and 40 μmol m^{-2} s^{-1} photons PAR (photoperiod of 12 h).

2.2. Treatments

Lichen and moss samples were incubated for 1 h with solutions of the commercial wood distillate "Distillato di Legno BioDea" produced by Esperia s.r.l. diluted with deionized water at concentrations 1:300 (corresponding to 0.333 mL L^{-1}), 1:500 (0.200 mL L^{-1}), and 1:700 (0.143 mL L^{-1}) and in deionized water only (control). The tested concentrations follow the usage dose recommended by the producer. The pure wood distillate used in this study is characterized by: pH 3.5–4.5, density 1.05 kg/L, high content of acetic acid (2.0–2.3%), phenols (2.90–3.02 g/kg) and polyphenols (23–26 g/kg) (BioDea, 2020). The acidity of the solutions prepared for this study was in the pH range 3.6–3.8.

2.3. Element accumulation

After 1 h of soaking, the samples were allowed to dry at room temperature for 24 h and left in a climatic chamber at constant conditions as above for additional three days. The samples were then stored at −20 °C until analysis. The samples (each of 200 mg of dry weight material) were mineralized with a mixture of 3 mL of 70% HNO_3, 0.2 mL of 60% HF and 0.5 mL of 30% H_2O_2 in a microwave digestion system (Milestone Ethos 900) at 280 °C and 55 bar. The concentrations of selected elements (As, Ba, Cd, Cr, Cu, Fe, Ni, Pb, Zn) were determined by inductively coupled plasma mass spectrometry (ICP-MS PerkinElmer – Sciex, Elan 6100). Results were expressed on a dry weight basis (μg/g dw). The quality of analytical procedures was checked with the certified Standard Reference Material NCS DC73350 'Leaves of Poplar', which indicated recoveries in the range 93–105%. Analytical precision was within 87% for As, 89% for Cr and 93% for all remaining elements. For each treatment, five replicates were measured. For comparison, the concentrations of the selected elements were also measured in pure wood distillate.

2.4. Physiological response

To assess the early effects of wood distillate on the vitality of *H. cupressiforme* and *X. parietina*, the treated samples were left in a climatic chamber with relative humidity up to 90% in order to maintain metabolic activity for 24 h, 48 h, and 96 h. The duration of the treatments and the experimental conditions allow detecting early effects on physiological responses of the target species (Vannini et al., 2018a).

2.4.1. Photosynthetic efficiency

Photosynthetic efficiency of hydrated thalli was assessed using a Plant Efficiency Analyser (Handy PEA, Hansatech Ltd, Norfolk, UK). The wet samples were dark-adapted for 10 min, lightened for 1 s with a saturating excitation pulse (1800 μmol s^{-1} m^{-2}) of red light (650 nm) and the fluorescence emission was recorded. The maximum quantum yield of primary photochemistry was inferred from chlorophyll *a* fluorescence emission: $F_V/F_M = (F_M\text{-}F_0)/F_M$, where F_0 and F_M are minimum and maximum chlorophyll *a* fluorescence and $F_V = (F_M\text{-}F_0)$ is the variable fluorescence. In addition, the performance index (PI_{ABS}), a global indicator of the photosynthetic performance was calculated to express the overall vitality of the samples (Strasser et al., 2000). The parameter PI_{ABS} combines in a single expression the three functional steps of the photosynthetic activity (light absorption, excitation energy trap-

ping, and conversion of excitation energy to electron transport), resulting in a very sensitive indicator of stress, suitable to be applied for physiological and environmental screenings. Moreover, the Normalized Difference Vegetation Index (NDVI) was measured by PlantPen NDVI 310 (Photon System Instruments, Czech Republic). NDVI is directly related to the photosynthetic efficiency, being related to the difference in plant reflectance in the visible and near-infrared wavelengths. Fifteen replicates were measured for each treatment and time.

2.4.2. Chlorophyll content

The chlorophyll content of dry samples (dried 24 h in a climatic chamber at constant conditions specified above), expressed as mg chlorophyll per m^2 of biological material (mg/m^2), was measured by Chlorophyll Content Meter-300 (Opti-Sciences CCM-300, Hudson, NH, USA) which infers the chlorophyll content based on reflectance/absorbance of radiation by chlorophyll. It provides accurate readings comparable to those determined using the dimethyl sulphoxide (DMSO) extraction method (Liu et al., 2019). Fifteen replicates were measured for each treatment and time.

2.4.3. Dehydrogenase activity

Dehydrogenase activity (dark respiration) was assessed by measurements of triphenyltetrazolium chloride (TTC) reduction to triphenylformazan (TPF). The reduction is directly linked to the activity of the mitochondrial respiratory chain (Ruf and Brunner, 2003), and thus to dehydrogenase activity. It is a good indicator of the viability of the samples (Bačkor and Fahselt, 2005). About 20 mg of dry material were incubated in 2 mL of 0.6% TTC and 50 mM phosphate buffer solution for 20 h in the dark. After this, the samples were removed and washed in distilled water. Formazan was subsequently extracted with 6 mL of ethanol at 65 °C for 1 h. Absorbance was red at 492 nm. Results were expressed as absorbance units/g (au/g dw). Five replicates were measured for each treatment and time.

2.4.4. Antioxidant activity

Antioxidant activity was assessed using the DPPH assay which is considered as an accurate method to evaluate radical scavenging activity of antioxidants (Kedare and Singh, 2011; Prakash, 2001). The assay is based on the measurement of the scavenging capacity of antioxidants towards a stable free radical α, α-diphenyl-β-picrylhydrazyl (DPPH). About 50 mg of dry material were homogenized in 1 mL of ethanol at 80% and then centrifugated at 20 000 rcf for 5 min. 100 μL of the moss extract (50 μL in case of lichen extract), were added to 1 mL of a DPPH solution (3.15 mg of DPPH in 100 mL of methanol at 80%), vortexed and left incubated in dark for 1 h. The blank contained 1 mL of methanol at 80% and 100 μL (or 50 μL in case of blank for lichen) of ethanol at 80%. The control contained 1 mL of DPPH solution and respective volume of ethanol at 80%. The free radical scavenging activities of the samples were expressed as percentages using the following formula: [1-(absorbance of the sample at 517 nm/absorbance of the control at 517 nm)] × 100.

2.5. Statistics

Data normality was preliminary checked with the Shapiro-Wilk test, and since most variables did not show a normal distribution, non-parametric tests were used. Outliers were identified by the Tukey test and removed from the dataset. The Mann–Whitney *U* test was used for horizontal comparison between treatments within the same time, and the Wilcoxon W signed rank test for vertical comparison between the same treatments at different times. In case significant differences emerged among control samples over time, the data were normalized as ratios to the respective control and the statistical analysis was performed again with the Mann–Whitney *U* test. All statistical computations were run using the free software R (R Core Team, 2020).

3. Results

3.1. Element content

The contents of the studied elements in pure wood distillate and in the lichen and moss samples after treatment with wood distillate solutions are shown in Table 1. Compared to the control, statistically significant ($p < 0.05$) uptakes emerged in lichens treated with 1:700 solutions in the case of Cr and Fe (up to 17%), 1:500 in the case of Ba, Cr, Fe and Pb (up to 36%), Ni (101%), 1:300 in the case of Pb (19%) and in mosses treated by 1:700 and 1:500 solutions in the case of Pb (up to 15%). Statistically significant releases of As in moss samples were detected after 1:700 and 1:500 treatments (up to 54%) and in the case of Ba after 1:300 treatment (11%). However, the results do not show any clear pattern of uptake or release with increasing concentration of wood distillate. Moreover, all measured concentrations are very low and do not indicate any biological and ecotoxicological problem.

3.2. Physiological response

The results of the treatments with wood distillate solutions on lichen and moss samples are shown in Fig. 1 (photosynthesis-linked parameters: F_V/F_M, $PI_{(ABS)}$, chlorophyll content, NDVI) and Fig. 2 (dehydrogenase and antioxidant activity).

Overall, there was no evidence of a negative effect of wood distillate in the lichen *X. parietina*. The conventional physiological indicator F_V/F_M was fairly stable comparing treatments in time, with occasional changes at concentrations 1:300 and 1:500 after 48 h and 1:700 after 96 h (Fig. 1A). The other physiological parameters (global photosynthetic performance $PI_{(ABS)}$, chlorophyll content, NDVI, dehydrogenase and antioxidant activity) did not show any temporal change nor concentration effect (Figs. 1B,C,D; 2A,B).

On the other hand, the influence of wood distillate treatments on the physiology of the moss *H. cupressiforme* was more evident. The treatments induced statistically significant variations of the photosynthetic parameters F_V/F_M and PI_{ABS} in time (Fig. 1A and B). However, based on the weakly decreasing values of F_V/F_M and PI_{ABS} normalized to their respective controls (Fig. 3), the results suggested only a weak influence on moss photosynthetic activity and the samples remained overall healthy. The rest of the physiological parameters tested did not show any response in time.

Comparing the different wood distillate concentrations, the influence on the photosynthetic parameters is pointed out by decreasing values of F_V/F_M and $PI_{(ABS)}$ when higher concentrations were used (Fig. 1A and B). Reduction of TTC to TPF, representing dehydrogenase (respiratory) activity, was not enhanced after the treatments (Fig. 2A). On the other hand, radical scavenging activity of antioxidants significantly increased with higher concentrations of wood distillate as suggested by DPPH assay (Fig. 2B).

4. Discussion

The average concentrations of all measured elements were well within the range commonly found in lichen and moss samples from unpolluted environments (Bergamaschi et al., 2004; Zechmeister et al., 2011) suggesting that wood distillate at the tested concentrations does not pose environmental problems to the studied cryptogams in terms of bioaccumulation of toxic trace elements. In fact, the measured concentrations were so low that the variations detected, even when statistically significant compared to controls, were most probably determined by the intrinsic variability of the samples (Loppi et al., 2019).

Noteworthy, acetic acid, one of the main components of wood distillate, was found to influence adsorption capacities for heavy metals

Table 1
Element concentration (median ± median absolute deviation, μg/g dw) in the lichen *Xanthoria parietina* and the moss *Hypnum cupressiforme* after incubation with deionized water (control) and 1:700, 1:500 and 1:300 wood distillate solutions. Statistically significant differences ($p < 0.05$) are indicated by different letters. Element concentration (μg/L) in pure wood distillate is also shown.

Xanthoria parietina					
Element	**As**	**Ba**	**Cr**	**Cu**	**Fe**
Control	0.464 ± 0.016	10.0 ± 0.5 a	3.09 ± 0.21 a	6.12 ± 1.07	817 ± 53 a
1:700	0.438 ± 0.029	11.6 ± 0.5 a,b	3.42 ± 0.12 b	5.77 ± 0.31	972 ± 66 b,c
1:500	0.500 ± 0.014	12.7 ± 0.9 b	3.92 ± 0.22 b	6.72 ± 0.76	1146 ± 9 b
1:300	0.522 ± 0.034	10.6 ± 0.3 a	3.54 ± 0.29 a,b	7.28 ± 1.35	929 ± 78 a,c
Element	**Cd**	**Ni**	**Pb**	**Zn**	
Control	0.047 ± 0.004	1.48 ± 0.06 a	1.28 ± 0.08 a	89.3 ± 30.3	
1:700	0.042 ± 0.003	1.78 ± 0.32 a,b	1.53 ± 0.05 a,b,c	89.6 ± 4.7	
1:500	0.079 ± 0.047	1.92 ± 0.37 b	1.75 ± 0.01 c	94.9 ± 11.9	
1:300	0.047 ± 0.005	1.85 ± 0.18 a,b	1.59 ± 0.05 b	81.5 ± 29.7	
Hypnum cupressiforme					
Element	**As**	**Ba**	**Cr**	**Cu**	**Fe**
Control	0.230 ± 0.014 a	23.7 ± 0.4 a	3.13 ± 0.30	6.41 ± 0.76	827 ± 73 a,b
1:700	0.132 ± 0.022 b	24.3 ± 0.9 a	3.39 ± 0.14	6.65 ± 0.50	900 ± 60 a,b
1:500	0.108 ± 0.030 b	24.3 ± 0.6 a	3.19 ± 0.20	6.07 ± 0.63	915 ± 9 a
1:300	0.189 ± 0.013 a	20.9 ± 0.3 b	3.34 ± 0.02	6.57 ± 0.13	768 ± 27 b
Element	**Cd**	**Ni**	**Pb**	**Zn**	
Control	0.093 ± 0.005	4.33 ± 0.28	2.26 ± 0.13 a	15.5 ± 0.5	
1:700	0.075 ± 0.012	5.51 ± 1.51	2.51 ± 0.05 b	11.3 ± 0.7	
1:500	0.070 ± 0.006	3.79 ± 0.29	2.58 ± 0.16 b	12.9 ± 0.3	
1:300	0.081 ± 0.001	3.85 ± 0.32	2.10 ± 0.07 a	14.1 ± 0.1	
pure wood distillate					
Element	**As**	**Ba**	**Cr**	**Cu**	**Fe**
	7	63	38	426	32 600
Element	**Cd**	**Ni**	**Pb**	**Zn**	
	12	54	231	3 847	

and metalloids of common soil sorbents used for the treatment of contaminated soils, by decreasing pH and enhancing the removal of As, Cd and Pb cations in multi-metal systems depending on the concentration of the solutions (Sun et al., 2020). Moreover, the low pH of the treating solutions may influence the uptake of heavy metals in lichens as well as their effects on chlorophyll integrity (Garty et al., 1992). Therefore, it cannot be ruled out that the incubation of lichens and mosses in wood distillate solutions could have induced element release in some case, as likely suggested by our results for e.g. As, Fe and Pb. However, since our control samples reflected only low concentrations of heavy metals, this hypothesis should be specifically tested in future studies.

It is known that pyroligneous acid may act as a catalyst of plant cell growth and enzyme activation which enhance various physiological and biochemical processes in plants, e.g. photosynthesis and nutrient absorption (Grewal et al., 2018). Because photosynthesis is linked to several metabolic pathways, its alteration may reflect the physiological status and vitality of photosynthesizing organisms (Kalaji et al., 2016). In the case of lichens, the potential responses are related to photobionts (algae and/or cyanobacteria) which are embedded in the thallus and provide nutrient flux to the mycobiont. Alterations thus may have impact on an otherwise relatively stable and well-balanced symbiotic system (Beckett et al., 2008).

Each of the investigated photosynthesis-linked parameter refers to different processes of the photosynthetic apparatus which could be potentially altered by stress induction. The maximum quantum yield of primary photochemistry of PSII (F_V/F_M) has already been evaluated as suitable indicator for early stress effects on sample vitality and investigated for several stress factors, such as metals and metalloids (Chen et al., 2015; Paoli et al., 2013, 2014a; Pisani et al., 2011), nitrogen compounds (Munzi et al., 2010; Paoli et al., 2010), ozone excess (Vannini et al., 2018b), biocides and farmaceuticals (Vannini et al., 2018a, 2018c), or excessive irradiance (Gauslaa and Solhaug, 2000; Martínez-Abaigar et al., 2006). In this study, wood distillate is tested as a stress factor for the model cryptogams for the first time. In fully saturated, healthy and unstressed mosses, F_V/F_M has optimum values around 0.76–0.83, depending on the species (Proctor, 2003; Proctor and Bates, 2018); meanwhile in lichens, it should reach values in the range 0.6–0.76 (Jensen and Kricke, 2002). In our experiment, most of the measured values were in these ranges, reflecting a healthy status of the lichen and moss samples. Although few alterations of F_V/F_M were recorded, e.g. occasional changes along time or weak decreases in samples treated by more concentrated solutions, they do not show consistent patterns of negative or positive effects of wood distillate on the samples.

In this case, the photosynthetic performance index (PI_{ABS}) could shed light on the results in a finer scale because it is a more comprehensive parameter which reflects the functionality of both photosystems I and II, as indicated by several studies (Kalaji et al., 2012; Paoli et al., 2010, 2014c; Živčák et al., 2008). Nevertheless, an effect of wood distillate solutions on the photosynthetic performance of *X. parietina* has not been observed after selected short-term treatments and in the case of *H. cupressiforme*, only a weak variation has emerged. Also the NDVI index showed quite stable spectral reflectance of chlorophyll pigments in the lichen and moss. Given the lack of specific literature related to the effects of wood distillate on lichens and mosses, some useful considerations and comparisons can arise from similar experiments using higher plants. Chen et al. (2016), did not find any effect of 0.25 mL L^{-1} (1:4000) wood vinegar solutions on the photosynthesis of lettuce (*Lactuca sativa*) grown hydroponically, which was decreased only at concentrations of 1 mL L^{-1} (ca. 1:1000). Abdolahipour and Highchair (2019) reported that application of wood vinegar at concentrations higher than 2000 mg L^{-1} (ca. 1:500) decreases the photosynthetic activity of cucumber (*Cucumis sativus*).

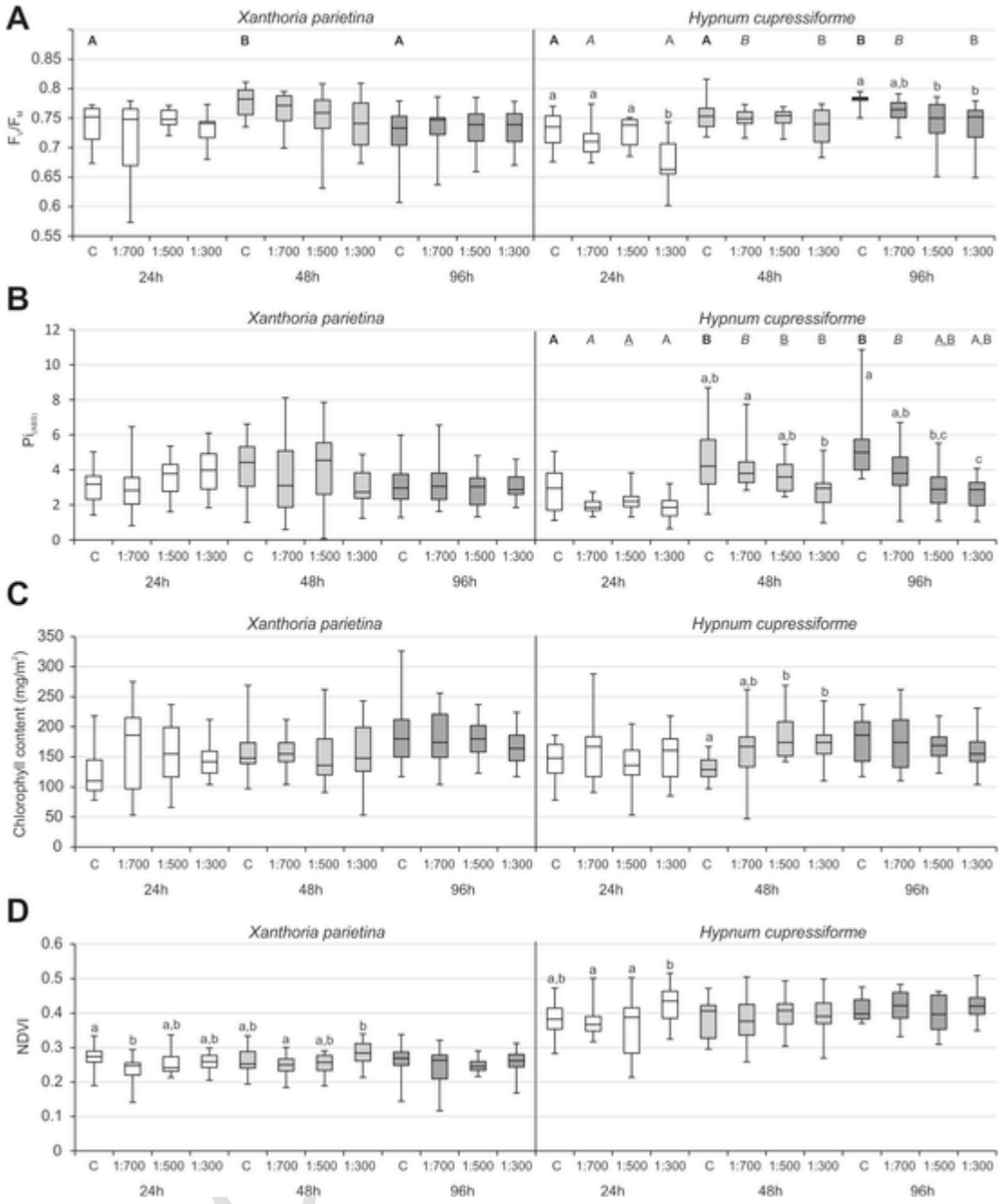

Fig. 1. Physiological parameters (median, quartiles, min, max): (**A**) maximum quantum yield of PSII primary photochemistry F_V/F_M; (**B**) photosynthetic performance index $PI_{(ABS)}$; (**C**) chlorophyll content; (**D**) Normalized Difference Vegetation Index NDVI of *Xanthoria parietina* and *Hypnum cupressiforme* treated with different concentrations of wood distillate (C = control, 1:700, 1:500, 1:300) after three measured periods (24, 48, 96 h). Statistically significant ($p < 0.05$) differences between treatments are indicated by different small letters. Statistically significant ($p < 0.05$) temporal differences are indicated by different capital letters: bold (for Control), italics (1:700), underlined (1:500), normal (1:300).

In general, it was suggested that the application of pyroligneous acid on leaves increases the chlorophyll content which consequently increases photosynthesis and synthesis of sugars and amino acids (Grewal et al., 2018). However, in case of wood distillate, we observed only an occasional increase of chlorophyll content in moss samples incubated with more concentrated solutions (1:300 and 1:500), while in the lichen *X. parietina*, chlorophyll content remained stable. Theerakulpisut et al. (2016) found that applications of wood vinegar at dilutions in the range 1:100–1:1000 reduce growth inhibition of rice (*Oryza sativa*) seedlings under salt stress and enhance the chlorophyll content of seedlings and 4-week-old rice plants, and concluded that treatments with wood vinegar modulated key physiological processes, leading to an improvement of ion homeostasis, mitigation of membrane damage, and prevention of chlorophyll degradation (Theerakulpisut et al., 2016). On the other hand, Mungkunkamchao et al. (2013) investigated the effects of wood vinegar supplied as soil drench and foliar spray in pot and field experiments on growth and yield of tomato (*Solanum lycopersicum*) and found out that there was no change in chlorophyll leaf content in response to the application of wood distillate (1:800) at any growth stage.

Dehydrogenase activity can be measured as indicator of the vitality of the samples, such as after treatments with heavy metals (Bačkor and Fahselt, 2005). As a rule of thumb, a decreased activity of respiratory dehydrogenases is negatively correlated with high element con-

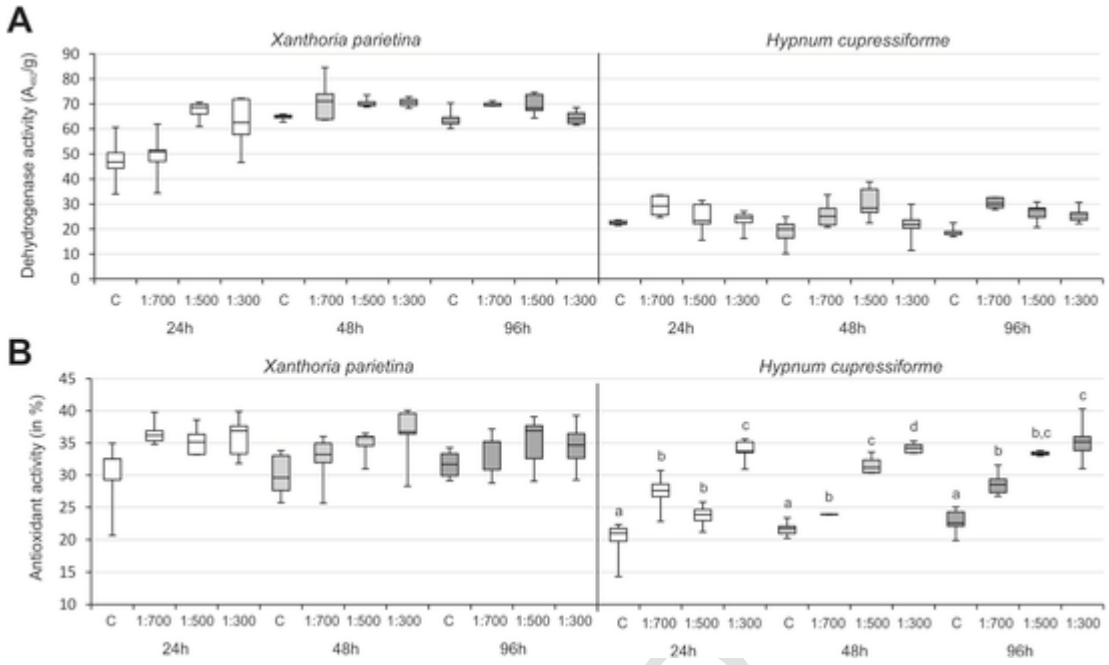

Fig. 2. Physiological parameters (median, quartiles, min, max): (**A**) dehydrogenase activity, **B**) antioxidant activity of *Xanthoria parietina* and *Hypnum cupressiforme* treated with different concentrations of wood distillate (C = control, 1:700, 1:500, 1:300) after three measured periods (24, 48, 96 h). Statistically significant ($p < 0.05$) differences between treatments are indicated by different letters. Statistically significant ($p < 0.05$) temporal differences did not emerge.

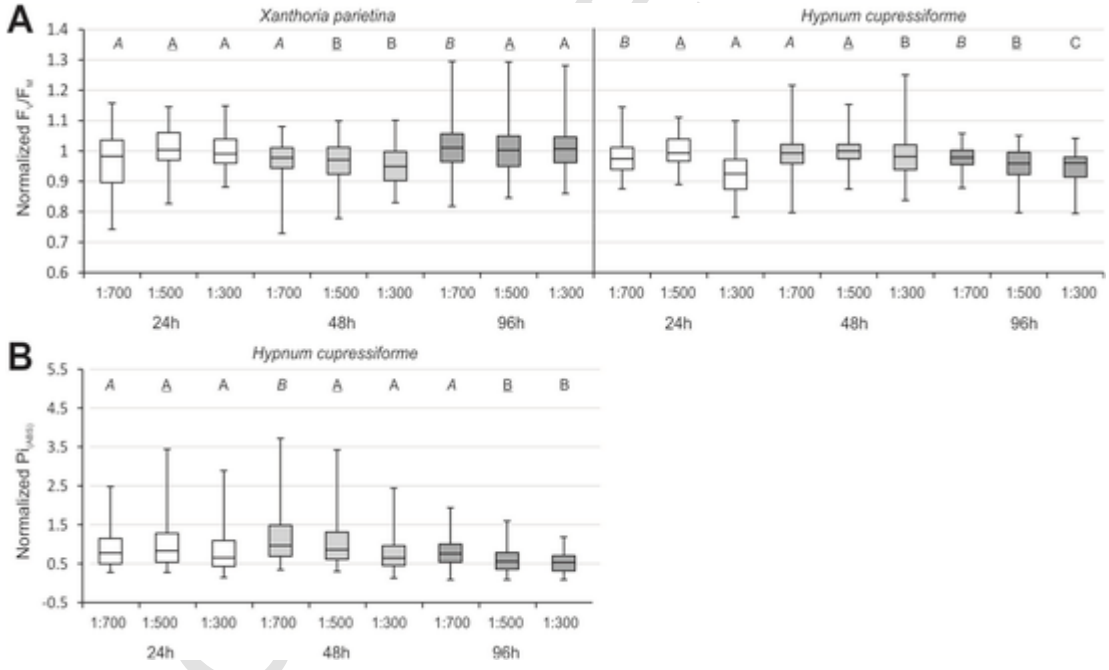

Fig. 3. Normalized values of physiological parameters: (**A**) maximum quantum yield of PSII primary photochemistry F_V/F_M; and (**B**) photosynthetic performance index $PI_{(ABS)}$. Axis *x* indicates concentrations of wood distillate (C = control, 1:700, 1:500, 1:300) after three measured periods (24, 48, 96 h). Statistically significant ($p < 0.05$) differences in time are indicated by different capital letters: bold (control), italics (1:700), underlined (1:500), normal (1:300).

tents (Pisani et al., 2011). However, within a certain degree, an increase could be linked to the functioning of other enzymes with antioxidant activity involved in the detoxification of heavy metals. Our results indicate that lichens and mosses were not significantly influenced by the treatment with any wood distillate solution. Cardelli et al. (2020) tested the potential toxicity or stimulation of pyroligneous acid on soil microbial community and enzymatic activity, including dehydrogenase activity. When applied in doses of up to 1% (note that our maximal solution was 0.33%), no negative effects on soil biology were observed and there was even an improvement of soil quality. Pyroligneous acid at higher doses (2% and 5%), induced a decrease in most enzymatic activities and microbial biomass, together with a loss of soil quality.

Pyroligneous acid has been also reported as active agent for the exhibition of antioxidant activity related to scavenging of free radicals before bio-macromolecules are damaged. Specific characteristics of pyroligneous acid produced from many types of biomass waste and using different pyrolysis methods result in potentially different power of an-

tioxidant activity (Petchpoung et al., 2020; Wei et al., 2010). Important sources of strong antioxidant power are especially phenolic compounds, e.g. Ortho-Catechol, Hydroquinone, 4-Methylbenzene-1,2-diol, 2,6-Dimethoxyphenol (syringol), Catechol, 3-Methoxycatechol (Liu et al., 2018; Loo et al., 2007; Yang et al., 2016). Our results suggest that in the moss *H. cupressiforme*, antioxidant activity increased progressively according to the dose of wood distillate supplied with the highest activity in 1:300 solutions. Similar to our results, an application of wood vinegar at concentration 1:250 enhanced an antioxidant property of tomato plant (Benzon and Lee, 2016). Wang et al. (2019) found that wheat seeds pre-soaked with wood vinegar developed plants with a higher ability to mitigate drought stress. Such plants showed a lower reactive oxygen species (ROS) and malonaldehyde (MDA) content, higher activity of major antioxidant enzymes and the production of specific stress proteins respect to plants, whose seeds were not pre-soaked in wood vinegar.

Many phenolic compounds can inhibit fungal hyphae development (Nyerges et al., 1975; Schlösser, 1994) or they bind the enzymes released by the fungal cells (e.g., during cell invasion). It is therefore reasonable that the mixture of phenolic compounds in wood vinegar could also contribute to its function as fungicide. As an example, Del Río et al. (2004) reported that phenolic compounds protect grapevine against fungal infections. However, based on our study, no significant changes were observed after short-term treatments in the lichen *X. parietina*.

Divergent responses to the treatments with pyroligneous acid can be attributed to several factors, such as ecological plasticity and stress resilience of different target organisms. In our experiment, lichen and moss samples were investigated in parallel and meanwhile lichens did not show significant changes, moss samples were occasionally altered. This could be explained either by the adaptability of the toxitolerant species *X. parietina*, or by a slower metabolism of lichens, which may show a delayed response to stress factors not exceeding critical levels after single short-term treatments. In fact, the response of lichens to stress factors can be not only species specific, but also time- and dose-dependent. As an example, when *X. parietina* was treated with various levels of ammonium nitrate and ammonium sulphate (Pirintsos et al., 2009), no effects were detected after single treatments, but repeated supplies led to a gradual decrease of photosynthetic activity after three weeks, until an almost complete inhibition of the photosystem II (Munzi et al., 2010).

Based on previous statements concerning the effects of pyroligneous acid, it is reasonable hypothesizing that contrasting responses could be related to: 1) biological attributes of the target organisms, 2) chemical composition of the applied pyroligneous acid, and 3) type of treatment (concentration, time of exposure, type of application).

5. Conclusion

A short-term exposure to wood distillate at working concentrations of 1:700, 1:500 and 1:300 did not cause negative impact on non-target sensitive organisms, such as lichens and mosses. Physiological parameters and the low bioaccumulation of toxic heavy metals indicated that the moss and the lichen treated with wood distillate remained healthy and did not show evident signs of negative alteration. Nevertheless, since in some agricultural practices it is suggested to repeat the applications of wood distillate several times in order to increase its effectiveness, the consequences of long-term wood distillate treatments should be also carefully assessed in future studies.

Author contributions

Zuzana Fačkovcová: investigation, formal analysis, writing - original draft. Andrea Vannini: investigation, writing - review & editing. Fabrizio Monaci: writing - review & editing. Martina Grattacaso: investigation. Luca Paoli: writing - review & editing. Stefano Loppi: conceptualization, supervision, funding acquisition, methodology, writing - original draft, writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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