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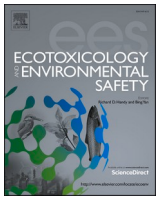

Cigarette butts enable toxigenic cyanobacteria growth by inhibiting their lethal fungal infections

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ABSTRACT

Cigarette butts (CBs), of which around 4.5 trillion are discarded annually, are one of the most common types of litter worldwide. CBs contain various chemicals, including metals, nicotine, and polycyclic aromatic hydrocarbons, which can leach into water and pose a threat to aquatic organisms such as cyanobacteria and chytrid fungi. Chytrids, zoosporic fungi that parasitize cyanobacteria lethally, play a crucial role in regulating cyanobacteria blooms by delaying or suppressing bloom formation. Despite the prevalence of CBs in the environment, the impact of their leachates on cyanobacteria-chytrid interactions is not well understood. We assessed the effects of CB leachate on the interaction between the toxigenic cyanobacterium *Planktothrix agardhii* and its chytrid parasite *Rhizophydium megarrhizum*. CB leachate inhibited cyanobacterial growth in uninfected cultures. Infection prevalence decreased at 0.2, 2, and 10 CB L^{-1}, with the two highest concentrations completely suppressing infection. Interestingly, at the highest CB concentration, cyanobacterial biomass in infected cultures was comparable to that of uninfected cultures not exposed to CB leachate, suggesting that the presence of chytrids mitigates the impact of the leachate. This study demonstrates that CB leachates are a potential environmental hazard that can enable cyanobacterial growth by inhibiting chytrid infections during epidemics. In addition, our research highlights the importance of assessing the effects of chemical mixtures, such as those leached from CBs, on multi-species interactions, such as host-parasite dynamics. These assessments are crucial to better understand the impact of pollutants on aquatic ecosystems.

1. Introduction

Approximately 5 trillion cigarettes are consumed worldwide each year, and about 90 % (4.5 trillion) are improperly discarded (Drope et al., 2022; Statista Search Department, 2024). The part of a cigarette left over after smoking is known as 'cigarette butt' (CB), and consists of a filter, wrapping paper, ash, and unburned tobacco (Poppendieck et al., 2016). CBs generate around 766 million kilograms of trash each year (UN, 2022), making them one of the most commonly littered item globally, accounting for approximately 25–40 % of the total collected litter (World Health Organization, 2022). The abundance of CBs in urban environments ranges from fewer than 1 to 38 CBs per m^2, and their prevalence depends on multiple factors, including pedestrian traffic, sweeping frequency, and weather conditions (Roder Green et al., 2014; Vasques Ribeiro et al., 2022).

Improperly discarded CBs represent a form of toxic waste that can enter water bodies through direct disposal or runoff. CBs contribute to microplastic pollution in aquatic systems, as over 90 % of cigarette filters are made from cellulose acetate fibers (Green et al., 2023a), with each filter containing around 12,000 to 15,000 fibers (Novotny and Hamzai, 2023). These fibers can detach and be released into the environment, with an estimated release of 100 fibers per CB per day, posing a threat to aquatic organisms through ingestion or release of chemical substances such as additives (Belzagui et al., 2021; Wright et al., 2015). CBs also contain a wide variety of compounds, including nicotine, polycyclic aromatic hydrocarbons (e.g., naphthalene, fluorene, phenanthrene), metals (e.g., Fe, Zn, Cu, Mn), and phenols (e.g., o-cresol, resorcinol, phenol), which can leach into water and soil (Acarer Arat, 2024). It has been estimated that a single CB has the potential to contaminate up to 1000 liters of water (Roder Green et al., 2014), highlighting their serious environmental impact. Chemicals leaching from CBs into water can adversely affect the survival, growth, mobility,

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and development of various aquatic organisms such as fish (Slaughter et al., 2011), mollusks (Booth et al., 2015), crustaceans (Freire Lima et al., 2021), and phytoplankton (Bonanomi et al., 2020; Lucia et al., 2023; Asensio-Montesinos et al., 2021). These effects have been observed at concentrations ranging from less than 1 CB L^{-1} to 25 CB L^{-1}. However, their impact on multi-organism systems, such as host-parasite interactions, is not well understood.

Phytoplankton, including microscopic organisms like green algae and cyanobacteria, are responsible for about half of the world's primary productivity. Hence, phytoplankton play a crucial role in aquatic environments as the cornerstone of several trophic webs (Falkowski, 2012). In recent decades, cyanobacteria have increasingly dominated phytoplankton communities due to eutrophication (Paerl et al., 2018) and other anthropogenic pressures (Ho et al., 2019). Proliferating cyanobacteria form blooms that can negatively impact the environment, economy, and health of organisms (Watson et al., 2015; Igwaran et al., 2024). For instance, cyanobacterial blooms change the color, odor, and taste of recreational and drinking water sources (Zhang et al., 2019) and induce an anoxic condition that can lead to aquatic die-offs. Additionally, some cyanobacteria produce metabolites, such as microcystin, cylindrospermopsin, and anatoxin-a, potentially toxic to humans and other organisms (Wood, 2016). These metabolites can be classified according to their primary target organ: microcystin and cylindrospermopsin are hepatotoxins, affecting mainly the liver, while anatoxin-a is a neurotoxin affecting the nervous system (Corbel et al., 2014). Cyanobacterial groups capable of producing these metabolites are referred to as toxigenic cyanobacteria.

Cyanobacterial growth is limited by a combination of abiotic factors, such as nutrient load and temperature (Yang et al., 2008), and biotic interactions, like parasitism and predation (Gerphagnon et al., 2015). Among these biotic factors, the most important parasites of phytoplankton, including cyanobacteria, are chytrids. Chytrids are zoosporic fungi from the phylum Chytridiomicota (Kagami et al., 2007), which produce lethal infections in phytoplankton. Thus, chytrids are pivotal in various ecological and evolutionary processes. For instance, chytrids can suppress or delay the formation of cyanobacterial blooms through their pathogenic effects (Gerphagnon et al., 2015). In addition, chytrids provide an alternative trophic link between primary producers and grazing organisms (Agha et al., 2016; Frenken et al., 2020). Given the crucial role of chytrid fungi and their phytoplanktonic hosts in the ecosystem, understanding how anthropogenic pollutants affect these host-parasite interactions is essential.

Anthropogenic pollutants can impact host-parasite dynamics in multiple ways, either benefiting the host by reducing parasitic infection or benefiting the parasite by increasing infection rates. The direction of the effect depends on different factors, including the mode of action of the pollutants and the duration of exposure. While many studies have focused on amphibians, crustaceans, fish, mammals, and mollusks (Sures et al., 2017), studies examining the ecotoxicological impacts of pollutants on chytrid-phytoplanktonic interactions remain scarce. Exposure to specific fungicides, pharmaceuticals, herbicides, and nanoplastics negatively affect chytrid infection prevalence (Ortiz-Cañavate et al., 2019; Raman et al., 2023; Schampera et al., 2021; Van den Wyngaert et al., 2014; Martínez-Ruiz et al., 2024). Similarly, polystyrene nanoplastics and diclofenac hampered the host growth, while metolachlor promoted it (Raman et al., 2023; Schampera et al., 2021; Martínez-Ruiz et al., 2024). Evaluating the effect of common pollutants, such as chemical mixtures leached from CBs, on phytoplankton-chytrid interactions could deepen our understanding of the impact of anthropogenic pollutants on phytoplankton disease dynamics and, more broadly, on aquatic ecosystems.

Conventional ecotoxicity tests typically focus on the effects of single pollutants on individual species. However, it is crucial to evaluate the toxicity of complex mixtures of pollutants on multi-species systems, such as host-parasite interactions, as pollutants in the environment are typically found in mixtures. This provides a more accurate reflection of real-world conditions and allows a deeper understanding of how pollution impacts aquatic ecosystems. Therefore, our study aimed to investigate whether CB leachate alters the dynamics of a cyanobacterium-chytrid fungus host-parasite system by evaluating its effects on cyanobacterial growth and the prevalence and intensity of chytrid infection.

Based on the existing literature on the effects of CBs on individual species and the known impacts of other anthropogenic pollutants on phytoplankton-chytrid interactions, we hypothesized that 1) CB leachate inhibits cyanobacterial growth in both the presence and absence of the parasite, and 2) CB leachate negatively affects chytrid prevalence. Both hypotheses emphasize the potential ecological impact of CB pollution on aquatic systems.

2. Materials and methods

2.1. Preparation of CB leachate stock solution

Self-rolled CBs with cellulose acetate filters and rolling paper from a popular German brand of smoking accessories were obtained from volunteers. Each cigarette contained an average of 0.45 ± 0.03 g of tobacco. Volunteers smoked the cigarettes until 1 cm from the edge of the filter and stub them out gently. CBs were collected over two weeks and stored in glass jars in a freezer until the CB leachate was prepared. The leachate stock solution was prepared by soaking CBs in distilled sterile water and kept in the dark at room temperature (approximately 18°C) with gentle agitation for 24 h. The resulting solution contained rolling paper, filters, and other particulate matter from the CBs. After soaking, the largest particles were removed with tweezers, followed by sequential filtration with 30 μm and 5 μm nylon mesh filters, and finally through 1.2 μm glass fiber filters to avoid rapid clogging. The leachate stock solution had a final concentration of 100 CB L^{-1} and was used immediately for toxicity testing. Samples for the chemical quantification were collected under sterile conditions and stored at 4°C until analysis.

2.2. Cyanobacteria and chytrid strains and culturing conditions

We used the host-parasite system consisting of the toxigenic filamentous cyanobacterium *Planktothrix agardhii* strain NIVA-CYA630 (monoclonal, non-axenic) and its obligate chytrid parasite *Rhizophydium megarrhizum* strain Chy-Kol2008 (Sønstebø and Rohrlack, 2011). Infections by *R. megarrhizum* are lethal for the cyanobacterial host. Cyanobacteria cultures were routinely maintained in Z8 medium (Kotai, 1972) under a continuous light intensity of 20 μmol photons m^{-2} s^{-1} at 16 °C. Under these conditions, cyanobacterial cultures remain viable for over a month without collapsing. Chytrids cultures were maintained by transferring zoospores to uninfected cyanobacteria cultures every three weeks. For the experiment, temperature and light conditions were the same as for the routine cultivation.

2.3. Toxicity tests with CB leachate

We assessed the effect of different concentrations of CB leachate on cyanobacterium-chytrid host-parasite dynamics. Infected and uninfected cultures were exposed to CB leachate corresponding to three different concentrations of CBs. For simplicity, the concentrations are referred to and discussed as the number of CBs per liter. The selected concentrations were based on the range used in the literature (Green et al., 2023a; Lucia et al., 2023; Baran et al., 2020; Dobaradaran et al., 2021; Green et al., 2020), the prevalence of CBs in urban environments (Acarer Arat, 2024), and the estimation that a single CB can contaminate about 1000 L of water (Roder Green et al., 2014). The experiment consisted of 40 experimental units, including 2 conditions (infected and uninfected cyanobacteria) × 3 CB concentrations (0.2, 2, 10 CB L^{-1}) and 1 negative control (no CB) × 5 replicates (Fig. S1). The experiments were conducted in Falcon® cell culture 50 mL bottles with a final volume of 30 mL.

An exponentially growing cyanobacterial culture was infected 10 days before the start of the experiment to produce chytrid zoospores. The infected culture was then filtered using a sterile 5 μm nylon mesh and 3 μm polycarbonate membrane (Agha et al., 2018). The absence of cyanobacterial filaments in the zoospore suspension was confirmed through microscopic examination. Zoospore density was quantified with a Sedgewick Rafter chamber under an inverted microscope (Nikon Ti Eclypse). Throughout the two weeks before the experiment, cyanobacterial cultures were maintained as exponentially growing semi-continuous cultures. Twice per week and at the starting day of the experiment, the optical density ($OD_{750\ nm}$) of the cultures was adjusted to 0.05, which corresponds to approximately 10^4 filaments mL^{-1}. We incubated eight cultures with a final volume of 180 mL per culture. Four out of the eight flasks were infected with the purified zoospores suspension, reaching a final concentration of 750 zoospores mL^{-1} per flask. The cultures were further incubated for six days to allow the infection to establish.

On the day of the exposure before adding CB leachate, uninfected cultures were diluted to an $OD_{750\ nm}$ of 0.05, which corresponds to the exponential growth phase. Infected and uninfected cultures were pooled separately and distributed into the containers. All experimental units were incubated for seven days under the conditions described in Section 2.2. Samples were taken from each experimental unit on days 0, 1, 3, 5, and 7. On each sampling time, 1 mL of culture was fixed with acid Lugol and stored at 4°C. All samples' identities were blinded and randomized before analysis.

2.4. Recorded parameters

Cyanobacterial growth was quantified as the change of biovolume over time using an inverted microscope (Nikon Ti Eclipse). Volume was estimated by measuring the length of the filaments in 5 fields of a Sedgewick-Rafter chamber per individual sample and applying the following formula:

$$V = \pi r^2 \quad h$$

where r is the mean radius of cyanobacterial filaments (mean width divided by 2), and h is the mean calculated filament length. The mean radius of cyanobacterial filaments was calculated for each exposure concentration (0, 0.2, 2, and 10 CB L^{-1}) and condition (infected and uninfected), accounting for variations in filament width. The mean width of 10 random filaments per sample was measured. If the mean width did not differ between consecutive sampling times, a combined mean width value was used for that time frame within the same condition and exposure concentration. Dead cyanobacterium filaments due to chytrid infection (i.e., empty, translucent filaments) were not included in biovolume calculations. Biovolume quantification in the uninfected cyanobacterial cultures was used to disentangle the effects of CB leachate on chytrid infection from those on the cyanobacterial host.

To comprehensively evaluate the impact of CB leachates on the chytrid parasite *R. megarrhizum*, we recorded two direct measures of parasite fitness:

1.1. Infection prevalence: this measure indicates the proportion of infected individuals within a host population. It was calculated by determining the percentage of cyanobacteria filaments with encysted zoospores or sporangia after randomly examining 200 filaments. Infection prevalence reflects the susceptibility of cyanobacteria to parasite infection across various CB concentrations, indicating whether the host was more or less susceptible to infection. Additionally, it allows for the evaluation of parasite transmission, define as the transfer of the parasite from one host to another (i.e., the number of secondary infections resulting from a primary infection).

1.2. Infection intensity: this measure represents the number of parasites infecting a single host. It was determined by calculating the average number of parasites (encysted zoospores or sporangia) infecting a single filament after examining 50 infected filaments per sample. Counting the precise number of parasites infecting a single filament becomes challenging when more than six zoospores or sporangia are present due to their overlap. Therefore, infected filaments with six or more sporangia or encysted zoospores were categorized as having six infections.

Cyanobacterial biovolume and infection prevalence were quantified in all samples. Infection intensity was assessed on day 7, but only in control cultures and those exposed to 0.2 CB L^{-1}, as there was no detectable infection at 2 and 10 CB L^{-1} on day 7.

2.5. Chemical analyses of the CB leachate

The concentrations of metals, non-metallic elements, metalloids, nicotine, and cotinine were quantified in three individual samples of the CB leachate stock solution. Dissolved concentrations of Al, B, Ca, Cd, Cr, Cu, Fe, Hg, K, Mg, Mn, Na, Ni, P, Pb, S, Si and Zn were measured in membrane-filtered (0.45 μm cellulose acetate) and acidified (0.25 M HCl) samples by inductively coupled plasma-optical emission spectrometry (ICP-OES, Thermo iCAP 7600, Thermo Scientific, Dreieich, Germany).

For nicotine and cotinine quantification, the CB leachate stock solution was filtered using 0.22 μm regenerated cellulose syringe filters (Chromafil® Xtra RC-20/13, Macherey-Nagel) to remove particulate matter. Nicotine and cotinine were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 triple quadrupole mass spectrometer in positive ionization mode. The analytes were separated using a XBridge BEH C18 column (5 cm×2.1 mm, 3.5 μm, WATERS) with a XBridge BEH C18 VanGuard cartridge (5 mm×2.1 mm, 3.5 μm, WATERS). The mobile phase consisted of LC-MS grade H_2O (solvent A, LiChrosolv, Supelco) and LC-MS grade methanol (solvent B, ≥ 99.95 %, Roth) both acidified with 0.1 % formic acid (Fluka). The eluent gradient increased linearly from 10 % B to 95 % B in 15 min and was kept constant at 95 % B for 1 min at a flow rate of 200 μL min^{-1}. For nicotine, the parent mass $[M+H]^+$ was 163.1 *m/z* with two product masses of 117.0 *m/z* (quantifier) and 130.1 *m/z* (qualifier). For cotinine, the parent mass was 177.1 *m/z* with two product masses of 80.0 *m/z* (quantifier) and 98.1 (qualifier). Quantification was performed using external calibration standards from 0.5 – 100 μg L^{-1} and the Agilent MassHunter Workstation software.

2.6. Data analyses

Linear mixed models were used to test changes in cyanobacteria growth and infection prevalence caused by exposure to CB leachate at specific time points. This approach accounted for multiple samples taken from the same experimental unit at different time points and for repeated measurements within each sample (multiple observations), where applicable. Separate models were run for the biovolume of uninfected and infected cultures. All the models used in the present study are summarized in Table S1 and described below.

We considered a two-way interaction between CB concentration and time for both tested parameters. The conditional R^2 (proportion of total variance explained by fixed and random effects) and marginal R^2 (proportion of total variance explained only by the fixed effects) were estimated for each mixed model. Residuals distribution was examined visually to verify if they met the assumption of normality with homogeneous variance. If this assumption was not met, the data was log or square root transformed in the models to normalize the residuals.

The significance of the interaction in the biovolume mixed model was assessed using the log-likelihood ratio (LLR), comparing a model

with the interaction term to one without it. The model for infection prevalence without the interaction had a singular fit, preventing assessment of the significance of the interaction in this case. Power analysis was conducted for the biovolume linear mixed models to estimate the power of the interaction based on the observed effect size. However, due to a singular fit of the infection prevalence model without the interaction term, the statistical power could not be estimated for this model.

Additionally, we calculated the area under the curve (AUC) per experimental unit from the biovolume and infection prevalence plots. The AUC approach provides a single value that combines data from the entire incubation period rather than concentrating on individual time points. To ensure a consistent baseline for the AUC calculations, the data was normalized to the initial biomass value of each replicate, per treatment and condition. Linear models were run using the AUC of each parameter as the response variable and CB concentration as the explanatory variable. Residuals distribution was examined visually to verify if they met the assumption of normality with homogeneous variance. Besides, we evaluated the homoscedasticity of residuals with the Breusch Pagan test. Power analysis was calculated for the linear models to estimate the power of the test based on the observed effect size.

A linear model was used to analyze the effect on infection intensity. Normal distribution and homogeneous variance of the residuals were checked as described for the AUC models. Non-parametric linear models were run to assess the differences in chemical concentrations between the CB leachate and the distilled water used to prepare it.

2.7. Software for data processing, analysis, and visualization

Cyanobacterial biovolume was measured with NIS-Elements BR 4.5 software (Nikon®). Statistical analyses and figures were performed using the R packages described below in R Statistical language (version 4.2.1; R Core Team, 2022). Linear mixed models were run using lme4 (Bates et al., 2015), *posthoc* tests were performed with emmeans (Lenth, 2022), and data visualization for normality and homogeneous variance was done with ggResidpanel (Goode and Rey, 2019). Conditional and marginal R^2 for each mixed model was estimated according to Nakagawa and Schielzeth (Nakagawa and Schielzeth, 2013) with MuMIn (Bartoń, 2022). Random effect significance was evaluated with lmerTest (Kuznetsova et al., 2017). Breusch Pagan test was performed using lmtest (Zeileis and Hothorn, 2002). Power analysis was conducted using simr for the linear mixed models and pwr for the linear models (Green and Macleod, 2016; Champely et al., 2017). Figures were made with ggplot (Wickham, 2016), ggpubr (Kassambara, 2020) and cowplot (Wilke, 2020). Additionally, different packages from the *tidyverse* (Wickham et al., 2019) were used to import, export, tidying, and arrange data. R code used to generate the results is provided as Supplementary material. The graphical abstract was created using Inkscape free software (Project, 2020).

3. Results

3.1. Chemical analyses of CB leachate

We quantified the concentrations of macroelements (Ca, K, Mg, Na, P, S), trace elements (Al, B, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Si, and Zn), nicotine, and cotinine in the CB leachate to evaluate their release from 50 CBs in 500 mL of water, equivalent to a final concentration of 100 CB L^{-1}, after 24 h. All chemicals that were detected above the limit of quantification were significantly higher in the CB leachate than in the water used to prepare the leachate (Table 1). Among the macroelements, the highest concentrations were measured for K and Ca (mean values of 189.1 and 146.4 mg L^{-1}, respectively), and the lowest concentrations for Na and P (mean values of 14.3 and 8.2 mg L^{-1}, respectively). For trace elements, Mn and Zn were present in the highest concentrations (mean values of 803.3 and 221.0 μg L^{-1}, respectively), whereas Fe and Si were found in the lowest concentrations (mean values of 109.7 and 103.3 μg L^{-1}, respectively). The leachate also contained 60.1 mg L^{-1} of nicotine and 1.0 mg L^{-1} of cotinine. In summary, chemical analyses of the 100 CB L^{-1} leachate, corresponding to the concentration of CBs used to prepare the leachate stock solution, showed high levels of potentially toxic elements and compounds.

Table 1
Concentrations of macroelements, trace elements, and organic compounds in CBs leachate and in distilled water used for preparing the leachate. Values represent means ± standard deviations ($n = 3$).

Chemicals	CB leachate (100 CB L^{-1})	Water	Chemicals per CB[+]
Macroelements (in mg L^{-1})			
Ca*	146.4 ± 0.15	0.20 ± 0.0	1.5
K*	189.1 ± 0.56	< 0.2	1.9
Mg*	31.9 ± 0.08	0.01 ± 0.0	0.3
Na*	8.2 ± 0.22	0.08 ± 0.0	0.08
P*	14.3 ± 0.04	0.001 ± 0.0	0.1
S*	22.3 ± 0.06	< 1,0	0.2
Trace elements (in μg L^{-1})			
Al*	124.0 ± 7.8	< 20	1.2
B*	180.7 ± 0.6	26.0 ± 0.0	1.8
Cd	< 100	< 100	<1
Cr	< 100	< 100	<1
Cu*	111.0 ± 9.5	1 ± 0.00	1.1
Fe*	109.7 ± 4.7	< 10	1.1
Hg	< 100	< 100	<1
Mn*	804.0 ± 3.0	1 ± 0.00	8.0
Ni	< 100	< 100	<1
Pb	< 100	< 100	<1
Si*	103.3 ± 15.3	40 ± 0.00	1.0
Zn*	221.0 ± 5.2	< 10	2.2
Organic compounds (in mg L^{-1})			
Nicotine*	60.1 ± 11.9	< 0.0005	0.6
Cotinine*	1.0 ± 0.00	< 0.001	0.01

[+] Concentrations per CB were calculated based on the initial leachate concentration of 100 CB L^{-1}. *Indicates significant differences between CB leachate and water. Values with "<" symbol indicate concentrations below the limit of quantification.

3.2. Toxicity tests with CB leachate

Cyanobacteria biovolume increased over time in uninfected control cultures and in cultures exposed to 0.2 CB L^{-1}, with the highest biovolume recorded on day 7. In contrast, exposure to 2 and 10 CB L^{-1} significantly inhibited cyanobacteria growth (Fig. 1A). On the initial day of the experiment, the biovolume in cultures exposed to 10 CB L^{-1} was higher than in the other treatments, including the control. However, this biovolume remained constant throughout the experiment, indicating growth inhibition. Fixed effects (i.e., CB concentration and time) explained 58 % of the variance in our model (Table S2). Overall, cyanobacterial growth in uninfected cultures was lower when exposed to 2 and 10 CB L^{-1} compared to controls (AUC: 0 CB L^{-1} – 2 CB L^{-1}: $df = 16$, $t = 6.139$, $p = 0.0001$; 0 CB L^{-1} – 10 CB L^{-1}: $df = 16$, $t = 4.210$, $p = 0.031$, adjusted $R^2 = 0.7$, power = 0.63, Fig. 1B).

In control cultures and those exposed to 0.2 CB L^{-1}, cyanobacteria biovolume was controlled by chytrid infection, reaching the highest biomass on days 5 and 3, respectively. In contrast, cyanobacterial growth increased in cultures exposed to 2 and 10 CB L^{-1}, even in the presence of the parasite (Fig. 1A). Fixed effects (i.e., CB concentration and time) explained 66 % of the variance in our model (Table S2). Cyanobacterial growth in infected cultures was overall higher when exposed to 10 CB L^{-1} compared to the other treatments (AUC: 0 CB L^{-1} – 10 CB L^{-1}: $df = 16$, $t = -3.165$, $p = 0.028$, 0.2 CB L^{-1} – 100 CB L^{-1}: $df = 16$, $t = -3.602$, $p = 0.012$, 2 CB L^{-1} – 100 CB L^{-1}: $df = 16$, $t = -4.067$, $p = 0.005$, adjusted $R^2 = 0.5$, power = 0.32, Fig. 1B).

CB exposure hampered chytrid infection at all tested concentrations,

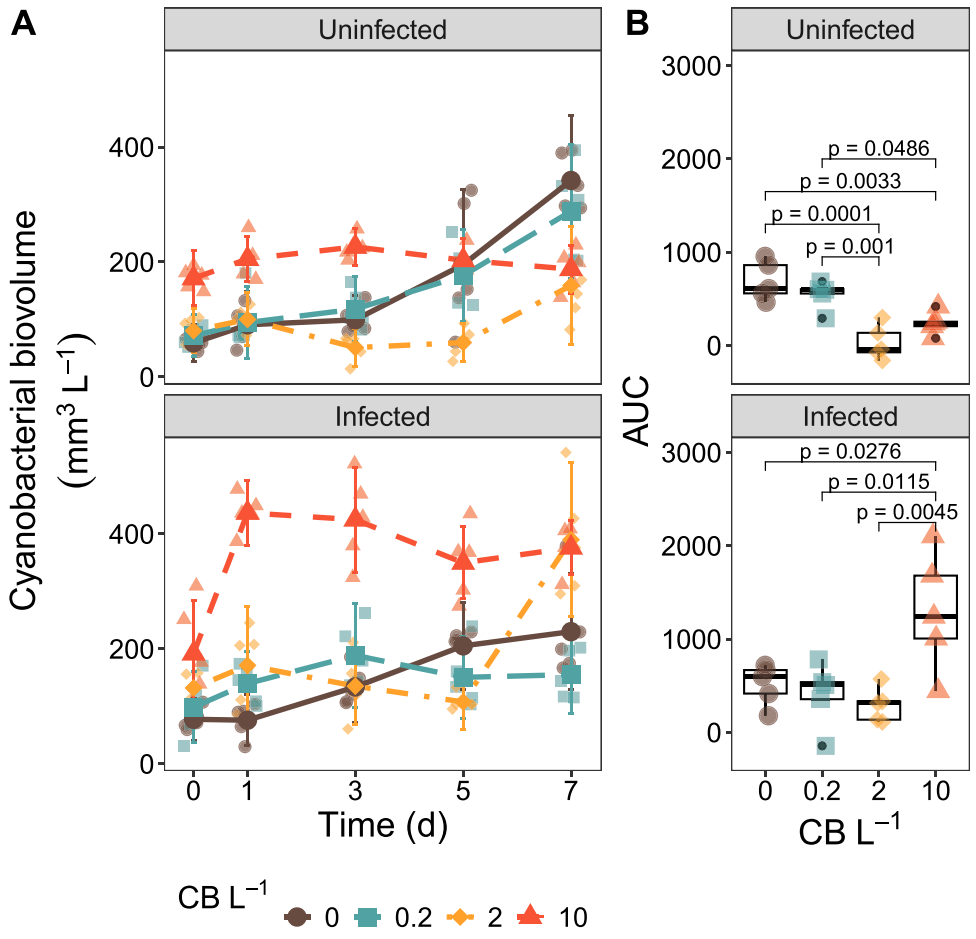

Fig. 1. Cyanobacterial growth in cultures exposed to CB leachate for seven days. A) Growth curves in uninfected (upper panel) and infected (lower panel) cultures. Mean values ± s.d. are shown ($n = 5$). B) Area under the curve (AUC) calculated from cyanobacterial biovolume in uninfected (upper panel) and infected cultures (lower panel). Data was normalized to the initial biomass value of each replicate per treatment and condition. Box indicates the upper and lower quartiles, the dark middle line indicates the median, the whiskers indicate 1.5 times the interquartile range and black points represent values outside this range ($n = 5$).

with complete suppression of the infection at 2 and 10 CB L^{-1} (Fig. 2A). Infection prevalence in cultures exposed to 2 and 10 CB L^{-1} was significantly lower from day 1 and 3, respectively. In cultures exposed to 0.2 CB L^{-1}, infection prevalence was significantly lower by the end of the experiment. Fixed effects (i.e., CB concentration and time) explained 95 % of the total variance in our model (Table S2) Overall, infection prevalence was significantly higher in the controls than in the cultures exposed to CB leachate (AUC: 0 CB L^{-1} – 0.2 CB L^{-1}: $df = 16$, $t = 2.949$, $p = 0.042$, 0 CB L^{-1} – 2 CB L^{-1}: $df = 16$, $t = 7.296$, $p < 0.0001$, 0 CB L^{-1} – 100 CB L^{-1}: $df = 16$, $t = 11.177$, $p < 0.0001$, adjusted $R^2 = 0.9$, power = 0.84, Fig. 2B).

Infection intensity was similar in both control cultures and those exposed to 0.2 CB L^{-1} ($df = 8$, adjusted $R^2 = -0.103$, $p = 0.7003$, Fig. 3). We could not evaluate the severity of the infection in cultures exposed to the highest tested concentrations due to the complete inhibition of the parasitic infection caused by the CB leachate.

4. Discussion

This study demonstrates that self-rolled cigarette butts (CBs) release a complex mixture of organic and inorganic chemicals into water, which can negatively affect cyanobacteria-chytrid host-parasite systems. Specifically, we found that exposure to CB leachate hindered cyanobacterial growth when parasites were absent. However, in the presence of chytrid parasites, cyanobacteria growth continued, as CB leachate inhibited chytrid infections. This suggests that the presence of the parasite reduced the inhibitory effect of CB-derived chemicals on cyanobacteria. Given the pivotal role of host-parasite interactions in ecological and evolutionary processes, this study deepens our understanding of how complex mixtures of anthropogenic pollutants, like CB leachates, can alter the structure and function of aquatic ecosystems.

Chemical analyses of the CB leachate evidenced the release of various metals, non-metallic elements, metalloids, nicotine, and cotinine into the water. Concentrations of Al, Cu, Mn, and Zn in the CB leachate produced in this study were within a similar range to those previously reported leaching from commercial and self-rolled CBs into water over a comparable time (Lucia et al., 2023; Cardoso et al., 2018; Quéméneur et al., 2020; Yang et al., 2023; Akhbarizadeh et al., 2021; Koutela et al., 2020). In contrast, Fe levels in our CB leachate were approximately half of the lowest concentration detected in previous studies (Lucia et al., 2023; Cardoso et al., 2018; Quéméneur et al., 2020; Akhbarizadeh et al., 2021). Concentrations of K, Mg, Na, and P were up to six times lower than those reported by Cardoso et al (Cardoso et al., 2018). Levels of Cd, Cr, Hg, Ni, and Pb were below our limit of quantification (i.e., < 100 µg L^{-1}), which is consistent with previous studies reporting low levels of these metals leaching from CBs (Lucia et al., 2023; Quéméneur et al., 2020; Akhbarizadeh et al., 2021; Koutela et al., 2020). Nicotine, the main alkaloid present in tobacco and one of the most abundant organic compounds leaching from CBs, has been reported at concentrations ranging from 0.07 to 3.5 mg L^{-1} per CB (Wright et al., 2015; Lucia et al., 2023; Venugopal et al., 2021; Dobaradaran et al., 2024; Richardot et al., 2023; Caridi et al., 2020). The nicotine levels per CB in this study were 0.6 mg L^{-1} per CB, which fall within this range. Cotinine, the main nicotine metabolite, was also

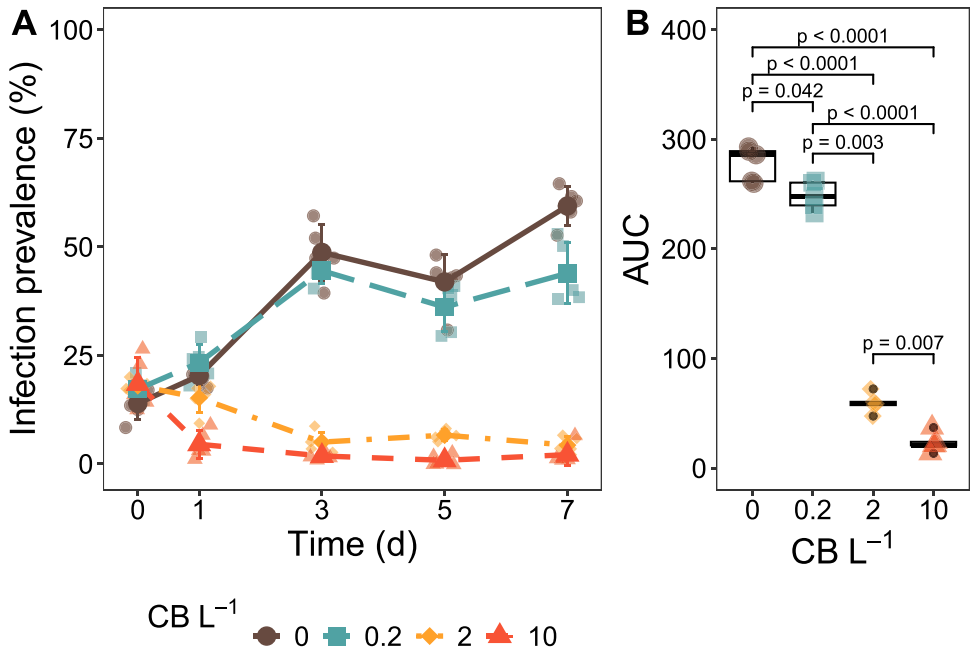

Fig. 2. Infection prevalence in cultures exposed to CB leachate for seven days. A) Percentage of cyanobacterial filaments infected with chytrid parasites. Mean values ± s.d. are shown ($n = 5$). B) Area under the curve (AUC) calculated from the infection prevalence over time. Box indicates the upper and lower quartiles, the dark middle line indicates the median, the whiskers indicate 1.5 times the interquartile range and black points represent any values outside this range ($n = 5$).

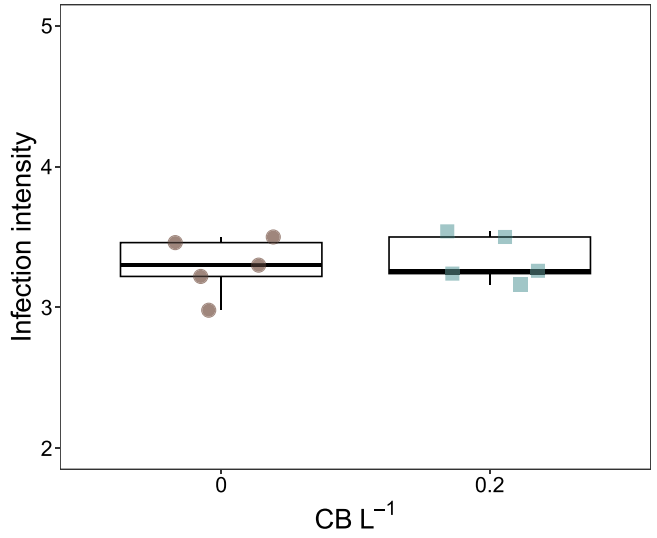

Fig. 3. Infection intensity in cultures exposed to CB leachate for seven days. Box indicates the upper and lower quartiles, the dark middle line indicates the median, the whiskers indicate 1.5 times the interquartile range and black points represent any values outside this range ($n = 5$).

present in concentrations comparable to those reported by Lucia et al (Lucia et al., 2023).

The concentrations of chemicals released from CBs can vary depending on several factors, including the number of CBs per volume of water (Lucia et al., 2023), the type of water used to soak CBs (seawater, distilled water, rainwater), soaking time, part of the cigarette discarded (i.e., the entire CB or just the filter without paper, ash, and leftover tobacco), cigarette brand, the tobacco's source and cultivation conditions, and the production of additional cigarette components (e.g., filters and paper) (Soleimani et al., 2022). Despite these variables, the chemical concentrations detected in our CB leachate were in line with those reported in other studies conducted under similar conditions, irrespective of the cigarette brand, type of water used for soaking, or the number of CBs used to produce the leachate. Thus, based on the chemical analyses, we can ensure the comparability of our results with other ecotoxicity assessments involving CB leachates.

CBs can enter water bodies either directly through improper disposal or indirectly via surface runoff. Upon contact with water, CBs begin to leach chemicals like nicotine and Zn, which are frequently detected in urban stormwater (Spahr et al., 2020; Wicke et al., 2021). Concentrations of nicotine can reach up to 18 μg L^{-1} (Masoner et al., 2019), while Zn levels can be up to 10,000 μg L^{-1} (Wicke et al., 2021). In surface waters, CBs continue to leach potentially toxic chemicals, with reported levels exceeding 100 μg L^{-1} for both nicotine and Zn (Wicke et al., 2021; Huerta-Fontela et al., 2008). These concentrations exceed the predicted no-effect concentrations (PNECs) for freshwater aquatic organisms, which are 0.4 μg L^{-1} for nicotine and 14.4 μg L^{-1} for Zn, according to the European Chemicals Agency (ECHA) (2023a; 2023b).

Given the varying concentrations of CBs used in our experiments, chytrids and cyanobacteria were exposed to chemicals, such as Cu, Zn and nicotine, at levels known to adversely affect aquatic organisms. For instance, Cu, Zn and nicotine inhibit the growth of *Raphidocelis (Pseudokirchneriella) subcapitata,* a widely used model for ecotoxicity assessment, at concentrations in the μg L^{-1} range (Al-Hasawi et al., 2020; Oropesa et al., 2017). While the toxicity of a chemical mixture can be theoretically estimated by examining the effects produced by the individual components, this approach can under- or overestimate the effects of the whole chemical mixture (Heys et al., 2016). Therefore, it was beyond the scope of our study to disentangle the individual contribution of each component. Instead, we considered any negative effects on chytrids and cyanobacteria to be the result of the entire chemical mixture and the potential interactions between its components.

CB leachate hindered cyanobacterial growth in uninfected cultures, consistent with reports that CBs inhibited the growth of other photosynthetic aquatic organisms like diatoms and green algae, at concentrations between 0.25 and 20 CB L^{-1} (Bonanomi et al., 2020; Lucia et al., 2023; Green et al., 2021; Oliva et al., 2021). Moreover, CBs have been shown to decrease the relative abundance of cyanobacteria in sediments at 25 CB L^{-1}, suggesting inhibition of this group (Quéméneur et al., 2020). In contrast, other studies have reported that CBs can stimulate

the growth of aquatic plants and green algae at concentrations of 1 and 2–20 CB L^{-1}, respectively (Oliva et al., 2021; Green et al., 2023b). To our knowledge, this study is the first to focus on the effects of CB leachates on cyanobacterial growth in the water phase, limiting the ability to compare this sensitivity with other cyanobacterial strains. Our findings suggest that complex chemical mixtures released by CBs can undermine photosynthetic microorganisms such as cyanobacteria, thereby affecting the structure of the phytoplankton community. Further studies are needed to determine the toxicity mechanism of chemical mixtures leaching from CBs in cyanobacteria.

CB leachate decreased infection prevalence, completely inhibiting it at the two highest concentrations tested. This inhibition prevented parasites from inducing typical host mortality, allowing the cyanobacterial host to grow. This aligns with previous studies showing decreased infection prevalence in phytoplankton-chytrid parasite systems exposed to other anthropogenic pollutants, including various fungicides (Ortiz-Cañavate et al., 2019), polystyrene nanoplastics (Schampera et al., 2021), and the herbicides diuron and metolachlor (Van den Wyngaert et al., 2014; Martínez-Ruiz et al., 2024). Given the negative effects of CB leachate, it is plausible that CBs in the environment could disrupt the mycoloop, impeding the transfer of carbon from inedible cyanobacteria to grazers (Agha et al., 2016; Frenken et al., 2020). Such disruptions may cause cascading effects throughout aquatic trophic webs.

Interestingly, cyanobacterial growth in infected cultures was not adversely affected by exposure to CB leachate, likely due to the inhibition of chytrid infections. In fact, cyanobacterial biomass in the infected cultures under exposure to CB leachate reached levels comparable to those of uninfected control cultures. This suggests that the presence of the parasite reduces the impact of the chemical mixture on the host, allowing cyanobacteria to thrive at the expense of the parasite. Hence, the presence of chytrid parasites might unexpectedly benefit cyanobacteria in polluted environments. Similar patterns of indirect benefits to hosts infected by parasites exposed to pollutants have been observed in other systems, such as fish-acantocephalan, fish-cestode and artemia-cestode interactions (Sures et al., 1994; Sánchez et al., 2016; Molbert et al., 2021; Brázová et al., 2021, 2012).

Further research is needed to determine the precise mechanisms behind the increased resistance of the host to CB leachate. Potential mechanisms include the uptake and accumulation of pollutants by parasites, which could reduce the bioavailability of these pollutants to the host, thereby reducing their negative effects (Sures et al., 1994; Brázová et al., 2021, 2012). In addition, parasites may enhance the resistance of the host to pollutants by altering general metabolism or antioxidant responses (Sánchez et al., 2016; Molbert et al., 2021). Another potential mechanism involves changes in cyanobacterial metabolism induced by chemical exposure, which may impair their ability to produce certain compounds essential for chytrids (Van den Wyngaert et al., 2014). Consequently, when chytrids infect cyanobacteria exposed to CB leachate, they may be unable to obtain the necessary nutrients for growth or to effectively locate their hosts.

Infection intensity was unaffected by 0.2 CB L^{-1}, while infection prevalence decreased at this concentration. Similarly, 10 mg L^{-1} of polystyrene nanoplastics reduced infection prevalence without affecting infection intensity (Schampera et al., 2021). This suggests that chytrids exposed to CB leachate remain metabolically capable of causing infections of similar severity as unexposed chytrids, as evidenced by the comparable infection intensity in cultures exposed to 0.2 CB L^{-1} and the control. However, the reduced infection prevalence implies that, although chytrids can still infect the host, their overall fitness to efficiently complete the infection cycle (i.e., to find the host, encyst, take up nutrients, and produce and release zoospores) is compromised, leading to a lower prevalence of infection over time. Additionally, exposure to the chemical mixture in the CB leachate might have impacted the phenotypic characteristics of the host, such as protein or lipid content, thereby decreasing the quality of available nutrients in the cyanobacterial host for chytrids and lowering their overall fitness. Consequently, while infection intensity remains comparable to that in unexposed cultures, infection prevalence is reduced due to less fit chytrids.

Altogether, our results partially support our initial hypothesis that CB leachate inhibits cyanobacterial growth in both uninfected and infected cultures. Moreover, our findings support the second hypothesis that CB leachate negatively impact chytrids, thereby disrupting host-parasite dynamics. Such disruptions can have wide-ranging effects on ecological and evolutionary processes involving chytrids and cyanobacteria. For instance, CB pollution might alter phytoplankton communities throughout the year. During seasons with low chytrid prevalence, CB leachates might inhibit cyanobacterial growth without the buffering effect of chytrids. In contrast, during periods of high chytrid prevalence, CB leachates might facilitate cyanobacterial growth by suppressing chytrid epidemics, which typically control cyanobacterial populations, potentially leading to harmful blooms. Therefore, CB pollution could contribute to the overgrowth of cyanobacteria.

Further studies should investigate whether CB leachates affect chytrids indirectly through their detrimental effects on the host or directly via the accumulation or incorporation of chemicals leached from CBs. Additionally, research is needed to determine whether the host's ability to proliferate in the presence of both the parasite and CB leachate exposure is due to increased general metabolism or reduced pollutant availability.

5. Conclusions

Our study demonstrates that the complex chemical mixture released from CBs poses an environmental hazard, disrupting host-parasite interactions and reducing parasitic infectious diseases. Given the sensitivity of chytrids to pollutants, they could serve as valuable indicators of environmental quality, similar to other parasites. This research highlights the detrimental effects of CBs, one of the most common forms of litter, on host-parasite systems, providing critical insights into the impact of complex chemical mixtures on aquatic multi-species communities. Our findings support the idea that CBs, a form of toxic waste, can threaten aquatic environments by negatively affecting microorganisms and their interactions. These insights are essential for guiding environmental policies aimed at managing CB disposal and reducing their adverse effects on aquatic ecosystems.

CRediT authorship contribution statement

Erika Berenice Martínez-Ruiz: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Nele Guttmann:** Writing – review & editing, Investigation, Conceptualization. **Justyna Wolinska:** Writing – review & editing, Resources. **Stephanie Spahr:** Writing – review & editing, Resources, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Erika Berenice Martinez-Ruiz reports financial support was provided by German Research Foundation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Supplementary figures and tables are included in Supplementary 1. All data and code are included as Supplementary material

(Supplementary 2–9).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117149.

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