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# Toxicity of metal cations and phenolic compounds to the bioluminescent fungus Neonothopanus gardneri

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ABSTRACT

Fungi play a key role in the soil ecosystem, where they occupy the first level of the food chain. Hence, they are considered suitable model organisms to conduct ecotoxicological assays for the evaluation of soil condition after proper soil extraction. Fungi bioluminescence-based bioassays are useful considering that their luminescence is an early toxicity endpoint and light emission a promptly detectable signal. In this paper, we describe a toxicological bioassay that relies on a 24-h variation of total light emitted by the mycelium of the bioluminescent fungus *Neonothopanus gardneri* when exposed to a toxicant. The current bioluminescent assay, which uses a fungus of the Omphalotus lineage, fills a gap covering all the representative species of bioluminescent fungi. Among the compounds tested here, Cd(II) showed the highest toxicity, followed by 4-nitrophenol, phenol and Cu(II), respectively. We also found that *N. gardneri* presents a predictable bioluminescence and growth pattern, and is highly sensitive to these compounds. The aforementioned characteristics offer valuable advantages and make *N. gardneri* the ideal candidate for toxicological studies with basidiomycetes.

## 1. Introduction

Among environmental pollutants, phenolic compounds and metal cations require special attention, since they occur in many different products/processes and are released on a massive scale in into the environment, causing highly negative impacts, especially to the biosphere (Arjoon et al., 2013; Igbinosa et al., 2013; Pradeep et al., 2015; Azimi et al., 2017; Duan et al., 2018; Li and Achal 2020; Rahman 2020; Roccuzzo et al., 2020). Several non-essential metals such as cadmium, lead and mercury are highly toxic even at very low concentrations. Metal cations are present in pigments, batteries, electronic devices, amalgams, fertilizers, pesticides, among many other industrialized products. They are also released from burning fossil fuels, mining, metallurgy and other industrial activities (Leyval et al., 1997; Gadd 2010). Metals do not degrade and can only speciate under biological or geochemical conditions. Hence, they can bioaccumulate in organisms of the soil food web and eventually give rise to serious ecological and possible health issues (Luoma and Rainbow 2005, Chojnacka 2010, Rahman 2020). Phenolic compounds can be found in the transformation pipeline of many industrial products (Santos and Linardi 2001; Lee et al., 2006). Due to their potential toxicity to microorganisms, plants and animals, phenolic compounds are defined as priority pollutants (Vera-Avila et al., 1996, Igbinosa et al., 2013). These compounds can be decomposed by bacteria and fungi, although many are persistent and harmful to the environment.

The toxic effects of metals and phenolic compounds to eukaryotic organisms include their direct interaction with biomolecules and subsequent modification, denaturation or inactivation of key enzymes (Appel 1993; Gadd 1993; Xu 1996; Baldrian 2003; Michalowicz and Duda 2007). Moreover, these effects can trigger the uncoupling of mitochondrial oxidative phosphorylation, leading to the impairment of ATP biosynthesis (Gadd 1993; Belgers et al., 2009; Stevani et al., 2013; Ventura et al., 2020).

A plethora of analytical techniques is available to identify and quantify specific pollutants, but unfortunately, these techniques do not serve to evaluate the bioavailability and/or toxicological effects of pollutants. Decomposer microorganisms such as fungi are on the first level of the food chains, cycling nutrients to species at higher trophic levels (Weitz et al., 2002; Mendes and Stevani 2010; Ventura et al., 2020). Therefore, deadly damage to fungi can affect the entire ecosystem, and hence, their potential bioanalytical role in ecotoxicology after proper extraction from soil.

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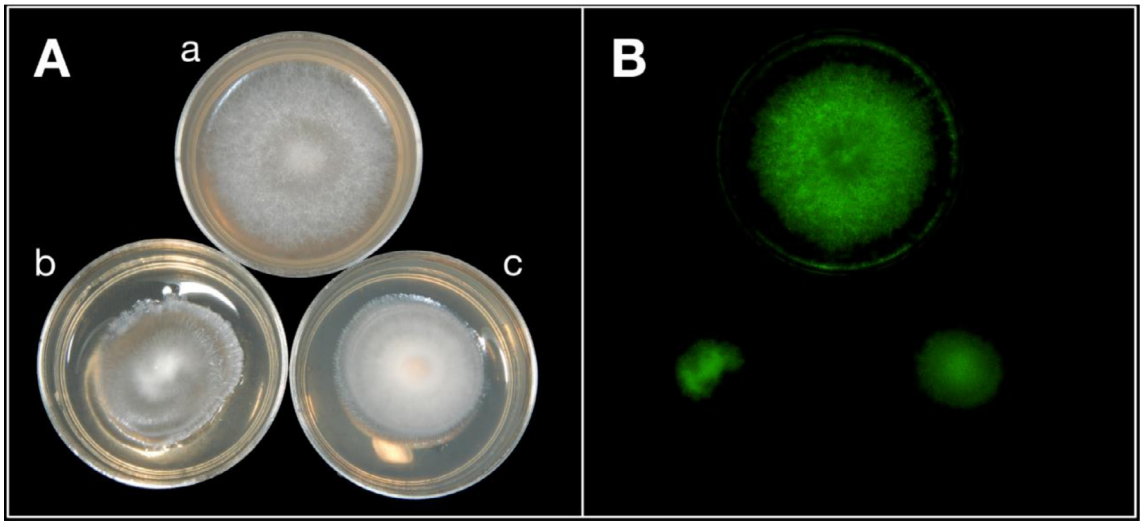


**Fig. 1.** Fourteen-day old mycelium of *Neonothopanus gardneri* (a), *Gerronema viridilucens* (b) and *Mycena lucentipes* (c) cultivated in the same conditions (A); the same Petri dishes in the dark (B).

In conventional bioassays using basidiomycete fungi, the toxicity of pollutants is usually assessed based on their inhibition of mycelium growth, which is determined by measuring the radial diameter or mycelial biomass (Weitz et al., 2002). On the other hand, bioluminescent bioassays are based on the decrease of light emission after mycelium exposure to the toxicant (Weitz et al., 2002; Mendes and Stevani 2010; Ventura et al., 2020). Luminescent methodologies are advantageous because the signal can be readily recorded; they are also sensitive, reproducible, less time-consuming and cost effective (Steinberg et al., 1995; Weitz et al., 2002; Mendes and Stevani 2010; Ventura et al., 2020). Moreover, as bioluminescence depends on molecular oxygen to occur and is therefore closely linked to respiratory activity, the decrease in bioluminescence emission is a prompt and early endpoint that signals injuries caused by toxicants to the fungus (Ventura et al., 2020). Directly or indirectly, the toxicant may act specifically or generally upon biological membrane, receptors and enzymes, finally causing light emission to decline. Potential biological targets of toxicants to fungi have been addressed by other authors (Mendes and Stevani 2010; Mendes et al., 2010; Ventura et al., 2020). The mechanism underlying fungal bioluminescence was recently described (Kaskova et al., 2017; Kotlobay et al., 2018).

All bioluminescent fungi are basidiomycetes (Desjardin et al., 2008; Stevani et al., 2013). Furthermore, as some bioluminescent basidiomycetes are phytopathogens (e.g., *Armillaria mellea* and *Mycena citricolor*) (Desjardin et al., 2008; Ventura et al., 2015), tailored bioassays within this group of fungi could be advantageous for the development of new agricultural fungicides (Gadd 1993; Stevani et al., 2013; Ventura et al., 2020). It is interesting to note that toxicological data on white-rot basidiomycetes are still scanty, and only a few reports have appeared in the literature in recent decades (Leontievsky et al., 2000; Mendes et al., 2010; Mendes and Stevani 2010; Ventura et al., 2020).

In an attempt to expand the range of available bioluminescent-based fungal assays, covering different *taxa* in all four lineages of bioluminescent fungi (Desjardin et al., 2008), further experiments initiated with *Gerronema viridilucens* (Mendes et al., 2010; Mendes and Stevani 2010; Stevani et al., 2013; Ventura et al., 2020) were conducted with the much brighter *Neonothopanus gardneri* (Fig. 1). For the purpose of comparison we describe here the analytical advantage of *N. gardneri* (Omphalotus lineage) over A*rmillaria mellea* (Armillaria lineage), *Mycena citricolor* (Mycenoid lineage), and *Gerronema viridilucens* (Lucentipes lineage) as the best model organism for the evaluation of the toxicity of metal cations and phenolic compounds to basidiomycetes (Desjardin et al., 2008). The bioassay methodology developed earlier required only small changes to adapt it to *N. gardneri*. This is promising: other bioluminescent fungi may also be used in such bioassays, following a similar protocol. The toxicological bioassay with *N. gardneri* was performed using the most toxic metal cations and phenolic compounds reported in the literature, namely cadmium, copper, phenol and 4-nitrophenol (Weitz et al., 2002; Mendes and Stevani 2010; Ventura et al., 2020). Our findings allow us not only to evaluate the simplicity of the bioassay methodology (i.e., exposure time and toxicant diluent), but also its fungus-dependent analytical sensitivity.

## 2. Material and methods

### 2.1. Chemicals and solutions

Fungal culture media were prepared using food-grade agar, yeast extract (both from Sigma-Aldrich, USA) and food-grade sugarcane molasses (55.94% sucrose, from Usina São José da Estiva, Novo Horizonte, SP, Brazil). All the reagents were of ≥ 99% ACS purity grade and used as received. Stock solutions of phenol, 4-nitrophenol, 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, Triton X-100 (from Sigma-Aldrich, USA), $CdSO_4.8H_2O$, $CuSO_4.5H_2O$, $H_2SO_4$ (from Merck, USA), NaOH (from Synth, Brazil) and dimethyl sulfoxide (DMSO; from Synth, Brazil) were prepared in ultrapure deionized Milli-Q water.

### 2.2. Equipment

The materials, solutions and culture media were sterilized in an autoclave (Phoenix, model AB42) at 120 °C for 30 min and handled in a laminar flow hood (Pachane, model PCR T2.5). *N. gardneri* culture were kept in a climate chamber (Binder, model KBWF 240) at 30 °C and 80% humidity. Distilled and ultrapure water was obtained using a Milli-Q Direct 16 water purification system (Merck-Millipore). Bioluminescence (BL) measurements were taken in a microplate luminometer (Tecan, model Infinite M200) with the integration time set to 100 ms per well.

### 2.3. Neonothopanus gardneri isolate and culture

*Neonothopanus gardneri* isolates (MycoBank MB519818) were first obtained in 2008 from fruiting bodies collected in Fazenda Cana Brava in the municipality of Altos, state of Piauí, Brazil. Samples were deposited in the herbarium of the Instituto Botânico, located in São Paulo, SP, Brazil (culture number SP416342). Store cultures for inoculation

were maintained under predetermined conditions (Ventura et al., 2015), which were optimized to obtain a more predictable and reliable stationary light emission intensity. In summary, the mycelium was grown for 14 days in 100 mm Petri dishes at 30 ˚C on a non-buffered agar (2%, pH 6) medium containing 1% sugarcane molasses and 0.02% yeast extract (all w:w). Freshly prepared cultures were then transferred to a climate chamber and kept in the dark to avoid any influence of ambient light on their bioluminescence profile (Berliner 1961; Bermudes et al., 1990; Oliveira et al., 2015).

### 2.4. Fungal toxicological bioassay

The protocol used in the bioassay has been described by other authors (Mendes et al., 2008; Mendes and Stevani 2010; Stevani et al., 2013). Briefly, *N. gardneri* cultures were incubated in 35-mm Petri dishes for seven days, which is the time required for bioluminescence to reach a predictable stationary phase under adjusted conditions (Ventura et al., 2015). Cultures were exposed to atmospheric air by opening and closing the lids of Petri dishes for 15 s, and to 500 µL of individual aqueous solutions of Triton X-100 (0.05%, w:v), DMSO (0.1%, v:v) and 50 mM MES buffer with Triton X-100 (0.01% and 0.05%) to evaluate the effect of diluent in the dissolution of target compounds. To identify the more suitable diluent, the bioluminescence of cultures exposed only to atmospheric air (used as control) was compared to that of cultures exposed to diluent. The ideal exposure time was determined by measuring the bioluminescence from mycelium exposed to 500 µL of individual solutions of $CuSO_4.5H_2O$ and phenol (respectively, 50 mM and 10 mM), both prepared in ultrapure water. The pH of all the solutions was adjusted to 5.7 with NaOH or $H_2SO_4$ (0.10 M). In each case, light emission was measured in triplicate after 0.5, 1, 2, 4, 6, 8 and 24 h of exposure.

The toxicity of individual solutions of Cu(II) (0.01–25 mM), Cd(II) (0.001–0.5 mM), phenol (0.01–10 mM) and 4-nitrophenol (0.01–10 mM) was then evaluated. The bioluminescence of the mycelium in all the dishes was measured on the 7$^{th}$ day of incubation. The plates were then uncovered and 500 µL of control (MES 50 mM with Triton X-100 0.01% in ultrapure water, pH 5.7), or the abovementioned toxicant, were applied on the surface and spread over the mycelium by gently shaking the dishes for approx. 15 s. The initial bioluminescence was then recorded ($BL_{initial}$). The intensity of bioluminescence was measured again after 24 h of exposure ($BL_{final}$). During this time, the dishes were kept covered in the dark at 30 ± 2 ˚C. Bioluminescence inhibition ($BL_{inhib}$) was calculated based on the ratio of final-to-initial light emission [$BL_{inhib} = 100 - (100 \times BL_{final}/BL_{initial})$]. Each compound concentration was tested in triplicate.

### 2.5. Data analysis

Bioluminescence values were determined by adding up the 384 points measured by the microplate luminometer (Tecan M200) over the entire surface of the individual adapter where Petri dishes were placed (Stevani et al., 2013). The bioluminescence inhibition data were plotted as a function of toxicant concentrations. EC50 values were calculated by adjusting the curves with a sigmoidal model (Eq. (1), Origin 2016 software, OriginLab Corp.).

$$BL_{inhib} = \frac{BL^{min} - BL^{max}}{1 + \left(\frac{C}{EC50}\right)^{n}} + BL^{max} \quad (1)$$

where $BL_{inhib}$ is the bioluminescence inhibition; $BL^{min}$ and $BL^{max}$ are the minimum and maximum values of $BL_{inhib}$; C, the toxic compound concentration; and n, the Hill number (curve slope) (Mendes and Stevani 2010; Stevani et al., 2013). The amount of metal cation in aqueous ion complex forms was estimated using MINTEQ version 3.0 software.

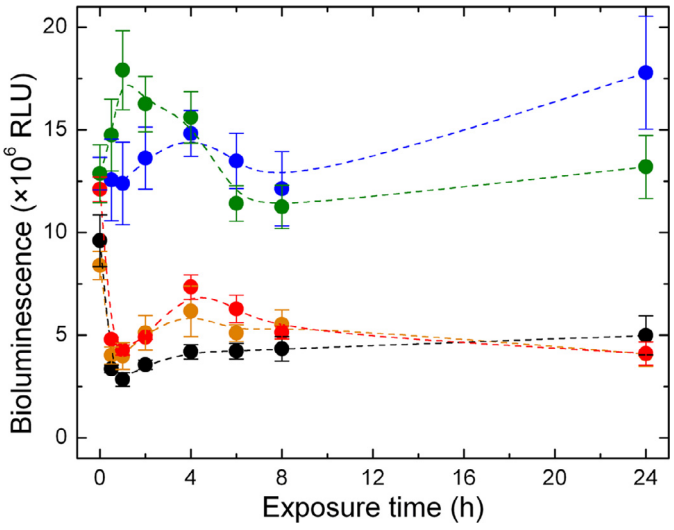


**Fig. 2.** Time course of bioluminescence in the presence and absence of different solutions applied on the surface of the mycelium of *Neonothopanus gardneri*. Control dishes containing mycelium without diluent, exposed only to ambient room air for 15 s (●), after applying 500 µL of aqueous solutions of 50 mM MES at pH 5.7 and 0.01% Triton X-100 (●), 50 mM MES at pH 5.7 and 0.05% Triton X-100 (●), 0.1% DMSO (●) or 0.05% Triton X-100 (●). Dotted lines indicate changes in the bioluminescence pattern over different exposure times. The number of replicates was $n = 9$ for control cultures and $n = 3$ for other samples.

## 3. Results and discussion

The suitability of diluent solutions used in toxicological assays is a very important parameter, since the solvent must be able to solubilize the toxicant without interfering with the bioluminescence itself. Three different aqueous diluent systems were studied here: Triton X-100, MES buffer and DMSO. Triton X-100 was chosen due to its ability to decrease the surface tension of water, thereby facilitating the homogeneous distribution of the toxicant over the mycelium. This diluent had been previously found to be innocuous to *Gerronema viridilucens* (Mendes and Stevani 2010). MES buffer, commonly used as a non-complexing biological buffer (Kandegedara and Rorabacher 1999), was tested to prevent pH changes that could affect both light emission and metal and phenol bioavailability. Conversely, DMSO was tested to solubilize lipophilic compounds, which is not achieved with Triton X-100 and MES solutions. In fact, the successful use of DMSO as a diluent has long been reported in bioassays (Gamliel et al., 1989; Haap et al., 2008).

Initial experiments were performed to ascertain the effect of diluents on the bioluminescence of *N. gardneri* (Fig. 2). Significant decrements in light emission were observed even after 30 min of exposure to solutions of 0.05% Triton X-100 and 0.1% DMSO. In both cases, bioluminescence was approx. 70% lower than that observed for the mycelium used as a control (Table S1). *N. gardneri* displayed a higher sensitivity to these diluents than other previously described fungi species, bacteria, protozoa and microalgae, which are reportedly non-responsive to the same DMSO concentration (Gamliel et al., 1989; Jay 1996; Bonnet et al., 2008). The toxic effect of DMSO on *N. gardneri* can be attributed to the solvent's interaction with cellular membranes, which changes their permeability, as described for some species of ascomycetes (Sharma and Sharma 2011). Moreover, the toxic effects of DMSO may have been the result of its direct contact with the mycelium instead of being previously diluted in the culture medium, as is common practice in non-bioluminescent fungal bioassays. It is worth noting that each culture dish in this bioassay is self-controlled, i.e., before the application of the toxic agent, the mycelium is the control with 100% light emission. This improves repeatability and decreases errors that normally occur when using a growth-based methodology (Mendes and Stevani 2010).

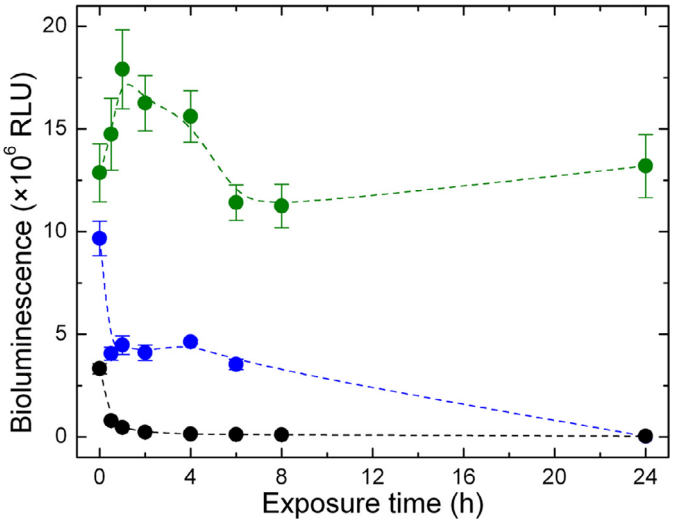


**Fig. 3.** Bioluminescence intensity as a function of exposure time and type of toxicant [Cu(II) or phenol] applied on the surface of mycelium of *Neonothopanus gardneri*. Light emission of cultures after applying 500 μL of 50 mM MES aqueous solution at pH 5.7 and 0.01% Triton X-100 (●, working solution), 50 mM Cu(II) (●) or 10 mM phenol (●). Dotted lines indicate changes in the bioluminescence pattern over different periods of exposure. The number of replicates was $n = 3$.

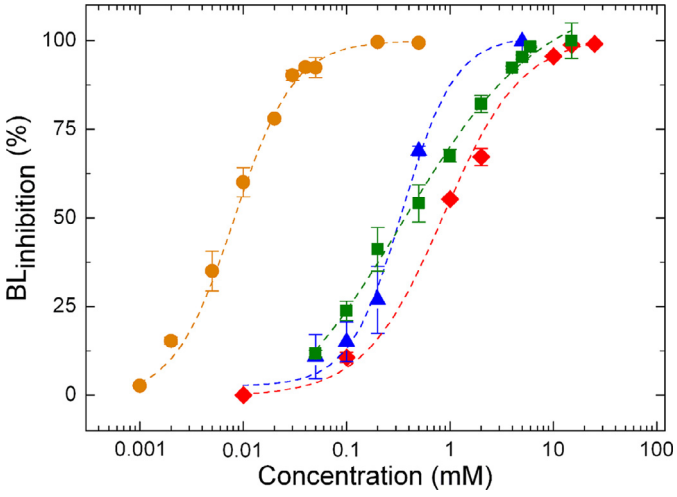


**Fig. 4.** Bioluminescence inhibition curves obtained from the luminescence assay with the fungus *Neonothopanus gardneri* in the presence of metal cations, cadmium (●) and copper (◆), and phenol (■) and 4-nitrophenol (▲). Seven-day-old mycelium and 24 h of exposure to the toxicant was used to perform the bioassay in triplicate ($n = 3$). The working solution was 50 mM MES aqueous-buffer at pH 5.7 containing 0.01% Triton X-100.

The decrease in the bioluminescence of the mycelium exposed to 0.05% Triton X-100 (0.80 mM) may be explained by the fact that its concentration is much higher than the critical micellar concentration (CMC, 0.22–0.24 mM) (Tiller et al., 1984). At 0.80 mM concentration, micelles are formed and no significant change in water surface tension is observed. The hydrophobic tail of the surfactant can interact with similar groups in proteins and lipids of cellular membranes, causing their rupture (Lodish et al., 2014).

The addition of MES does not seem to diminish the toxicity of the diluent, which is indicated by the intensity of bioluminescence, independently of its presence in Triton X-100 (0.05%) solutions (Fig. 2). As MES buffer does not exhibit any metal complexation, it was maintained in the presence of 0.01% Triton X-100 (0.16 mM, below CMC). In this case, bioluminescence intensity was higher than in other diluents, even when compared with the controls, for up to 8 h of exposure time. Hence, the solution of 0.01% Triton X-100 containing 50 mM MES at pH 5.7 was selected as the most suitable diluent for further bioassay tests.

It should be noted that the bioluminescence of *N. gardneri* mycelium follows a predictable and constant pattern (Ventura et al., 2015). The mean bioluminescence levels (obtained from the integral of light emission from the mycelium) on the 7$^{th}$ day of incubation under optimized conditions were similar throughout successive inoculations of 425 cultures in the closed Petri dishes used in seven different experiments performed over eight months. Measurements taken during distinct experiments using mycelium from different sources of inocula strengthen its predictable light emission pattern, which is a highly favorable feature in a bioluminescent bioassay.

Exposure time is also an important parameter for bioluminescence-based bioassays. In order to avoid underestimating toxicity due to insufficient exposure time, the ideal duration of experiments was considered to be the one that inhibited bioluminescence completely, i.e., the baseline light intensity. Exposure of the mycelium to phenol led to a 75% decrease in light emission within 30 min (Table S2, Fig. 3). Bioluminescence intensity remained constant between 4 and 8 h, with mean variations of less than 2% among all measurements, suggesting that the bioassay can be conducted within 6 h. The copper (II) solution showed approx. 50% bioluminescence inhibition after 30 min and up to 5 h of exposure. The maximum response of *N. gardneri* to Cu(II) exposure was attained after 24 h. These results indicate that the bioassay can be performed within a timeframe of 30 min to 24 h of exposure to pollutant. In view of this pattern, and for the sake of comparison with previous reports, 24 h was set as the standard exposure time for the bioassay to evaluate the toxicity of metal cations and phenolic compounds. This observation is in line with the bioassay methodology developed for the fungus *G. viridilucens* (Mendes and Stevani 2010; Ventura et al., 2020).

The bioluminescent assay was then performed in the presence of phenol, 4-nitrophenol, Cu(II) and Cd(II), under the previously optimized conditions for light emission (Ventura et al., 2015), diluent and exposure time (Table S3, Fig. 4). Based on EC50 values (Table S4), the toxicants were ranked in an increasing order of toxicity, as follows: phenol < Cu(II) < 4-nitrophenol < Cd(II).

The toxicity of phenolic compounds ranged from 1.3 μM of pentachlorophenol to *M. citricolor* up to 970 μM of phenol to *G. viridilucens* (Table 1). For all fungi tested, phenolic compounds bearing weakly acidic, hydrophobic and strongly electron-withdrawing groups such as nitro and chlorine proved to be the most toxic ones, which is consistent with our former results (Ventura et al., 2020). As recently reported, the toxicity of phenolic compounds can be explained by the uncoupling of ATP biosynthesis in mitochondria and can be predicted by a bivariate model, depending on the acid dissociation constant ($pK_a$) and the 1-octanol/water partition coefficient ($K_{ow}$) of the phenolic compound (Ventura et al., 2020). Basidiomycetes are reportedly very effective in the biodegradation of lignin, a macromolecule with a myriad of phenolic groups. This could exert significant resistance to phenolic compounds, as well as other organic pollutants. This behavior was observed in the basidiomycete *Trametes versicolor*, which showed a minimum two-fold higher inhibitory concentration for phenol than that observed for the brown rot *Coniophora puteana*, which produces lower amounts of laccase (Voda 2003).

Under the experimental conditions of the bioassay, the metals studied here were present mainly in solution as free cations, approx. 99.9% and 94.6% for Cd(II) and Cu(II), respectively. That is, at least before their application on the surface of mycelium, copper and cadmium would be in their most bioavailable form.

Toxicity of free metal cations can be reasonably predicted using a univariate model based on the metal cation covalent index ($X^2_m r$) (Mendes et al., 2010). The covalent index indicates the covalent bond of metal cations with biomolecules. Most toxic metal cations are the largest and most polarizable ones, increasing the probability of forming

**Table 1**
Comparison of effective median concentrations (EC50) obtained in toxicological bioassays in the presence of metal cations and phenolic compounds for different bioluminescent fungi species.

| Fungus[a] | Growth Medium | Toxicant | EC50 (μM) | Reference |
|---|---|---|---|---|
| *Neonothopanus gardneri* | agar | Cd(II) | 1.25 ± 0.07 | This work |
| | | 4-nitrophenol | 58 ± 8 | |
| | | Cu(II) | 160 ± 13 | |
| | | phenol | 395 ± 92 | |
| *Gerronema viridilucens* | agar | Cd(II) | 400 ± 20 | Mendes & Stevani (2010) |
| | | 4-nitrophenol | 300 ± 30 | |
| | | Cu(II) | 950 ± 30 | Ventura et al. (2020) |
| | | phenol | 970 ± 50 | |
| | | Zn(II) | 8,000 ± 333 | |
| *Armillaria mellea* | liquid | Cu(II) | 1.3 ± 0.3 | Weitz et al. (2002) |
| | | pentachlorophenol | 30 ± 3 | |
| | | 3,5-dichlorophenol | 83 ± 7 | |
| | | Zn(II) | 491 ± 70 | |
| *Mycena citricolor* | liquid | pentachlorophenol | 1.3 ± 0.1 | |
| | | Cu(II) | 35 ± 10 | |
| | | 3,5-dichlorophenol | 56 ± 2 | |
| | | Zn(II) | >1,377 | |

[a] Bioluminescence-based bioassays were performed in agar medium, with 24 h of exposure for *N. gardneri* and *G. viridilucens* and in liquid medium and 1 h of exposure time for *A. mellea* and *M. citricolor*.

stable non-labile complexes with biomolecules, especially those containing sulfur (Mendes et al., 2010). More polarizable cations such as Cd(II) and Cu(II) can easily interact with sulfur containing groups (e.g., thiols) in biomolecules and enzymes that are used to protect the organisms against reactive oxygen species (ROS, which contribute to oxidative stress) (Gadd 1993; Mendes and Stevani 2010). Copper and cadmium can also interact with fungal cell membranes, thus interfering with the solute cellular transport. In addition, both metal cations studied here can affect the permeability and composition of the membrane and bind to aromatic amino acid residues of enzymes (Baldrian 2003).

Paradoxically, copper cations are essential to organisms and are required in enzymatic activities related, for instance, to lignin degradation, which could explain a higher tolerance of *N. gardneri* to these metal ions. Moreover, several authors have stated that exposure to copper could stimulate the biosynthesis of laccases, which could contribute to melanin biosynthesis – a natural chelant produced by basidiomycetes (Henson et al., 1999; Baldrian 2003). It is likely that melanin – known to be biosynthesized from tyrosine – was produced in the present work in the form of brown droplets that were observed on the surface of the mycelium after 24 h of exposure to the most concentrated Cu(II) solutions. Other authors have reported the same observation, which indicates the possible biosynthesis of melanin as a fungal defense mechanism (Guillén and Machuca 2008).

Unlike copper, cadmium does not have a known biological role, even in small amounts. Its toxicity was higher than the other tested compounds, indicating that *N. gardneri* is less tolerant to this metal. The adverse result obtained with Cd(II) may be due to the lower adaption of *N. gardneri* to cadmium, which is not usually present in the natural habitat of this fungus. Cd(II) toxicity has been attributed to competition for binding sites of Ca- and Zn-dependent proteins and enzymes, direct inactivation reaction involving thiol-biomolecules, as well as alterations in the integrity of cell and organelle membranes (Gadd 1993; Baldrian 2003).

*N. gardneri* has been shown to be more sensitive to metal cations than *G. viridilucens* (Mendes and Stevani 2010; Ventura et al., 2020), but also more resistant than *A. mellea* and *M. citricolor* (Weitz et al., 2002) (Table 1). As expected, the accuracy of EC50 values for the same toxicant vary among different bioassays given the wide range of parameters involved in the analysis, such as the fungus species-specificity (including defense mechanisms), culture medium composition, exposure times and incubation conditions (e.g., temperature and pH of growth media).

*A. mellea* mycelium presented an EC50 of 1.3 μM for Cu(II), but a 30-fold lower toxicity was verified with *M. citricolor* (EC50 36 $\mu$M, Weitz et al., 2002), whereas the toxicity of Cu(II) to *G. viridilucens* was even lower: EC50 0.95 mM (Ventura et al., 2015). Copper cation toxicity to the non-bioluminescent basidiomycetes *Laetiporus sulphureus* and *Ophiostoma* sp. was also determined by conventional non-bioluminescent fungal bioassays based on the inhibition of mycelium growth in agar medium (Guillén and Machuca 2008). In these cases, these species showed distinct toxicities, with EC50 values of around 3 and 10 mM, respectively. In another conventional bioassay based on the biomass inhibition in liquid cultures of *Suillus variegatus* and *S. granulatus* exposed to Cd(II), the EC50 values obtained were 8 nM and 12.6 μM, respectively (Hartley et al., 1997). Zn(II) is the least toxic substance for *G. viridilucens, A. mellea* and *M. citricolor*. *N. gardneri* is also expected to show the same tendency.

On the other hand, results of fungal bioassays challenged with phenol or 4-nitrophenol are scanty. An evaluation of 4-nitrophenol toxicity to the ascomycetes *Aspergillus fumigatus, A. flavus* and *A. tamari* indicated EC50 values of around 0.5 mM after 20 days of exposure, and the inhibition of mycelium growth in liquid medium was adopted as endpoint (Bharathi et al., 2009). Another study conducted with 24 ascomycetes using the mycelium inhibition in liquid medium after 15 days of exposure to phenol resulted in EC50 values in the range of 1.1–9.0 mM (Guirald et al., 1995). Accordingly, the tolerance among fungi in those studies also tended to be higher for phenol than for 4-nitrophenol. That was the reason we decided to test the toxicity of these two phenol compounds to *N. gardneri*.

Bioassays using microorganisms in liquid media favors a greater bioavailability of pollutants, since it eliminates possible interactions of compounds with agar, which is normally used as a gelling agent. Even upon direct application of toxic chemicals on mycelium, as was done in this work, aqueous solutions diffused through agar over 24 h of exposure, thus favoring interactions between agar and pollutants and hampering their bioavailability (Ventura et al., 2020). This fact may explain the higher bioassay sensitivity in liquid medium. Additionally, the bioluminescent mycelium used in bioassays in liquid medium grows in globular shapes (3–5 mm diameter), thus increasing the surface area of the interaction between the fungus and toxic chemicals and leading to higher measurable toxicity. Last but not least, a hypothesis to explain the higher sensitivity of bioassays with bioluminescent fungi (i.e., *A. mellea* and *M. citricolor*) in liquid medium is that that the globular mycelium is removed and washed before exposure to the aqueous solutions of tox-

icants (Weitz et al., 2002). This could have prevented further interactions between the toxicants and nutrients present in the liquid growth medium.

Considering the usual fungal growth in soil, the methods developed in agar culture medium mimic the natural habitat of these organisms (Gadd 1983; Hartley et al., 1997; Mendes and Stevani 2010; Stevani et al., 2013). From this point of view, bioassays in agar are more suitable for terrestrial organisms, since their differentiation and biological responses are closer to what is expected in soil.

Thanks to the similar experimental conditions used for bioassays with *G. viridilucens* and *N. gardneri,* the results obtained with these two fungi, as well as their EC50 values, are fairly comparable. *N. gardneri* is more sensitive to metal cations and phenolic compounds than *G. viridilucens*. One of the reasons for this difference probably lies in the adaptations of *G. viridilucens* to the habitat where it can be found. This fungus was isolated from fruiting bodies collected in Atlantic Forest, Iporanga, SP, Brazil, in the proximity of a former lead mining site (Cotta et al., 2006). Therefore, it is reasonable to assume that *G. viridilucens* is better adapted to higher concentrations of non-essential metals than *N. gardneri*, which grows in Palm Forest (Mata dos Cocais) in the state of Piauí, northern Brazil, a region with no history of metal contamination. As for phenolic compounds, differences in sensitivity were lower, possibly due to the common features of basidiomycetes to secrete non-specific lignolytic enzymes, which can degrade phenols and other organic pollutants.

## 4. Conclusion

The less common use of soil microorganisms than of aquatic microorganisms to assess contaminant toxicity and the poor accuracy of different bioassays strengthens the argument for the need to extend studies to terrestrial species in order to better understand their development and biological responses. Fungi are of special interest in this respect because of their key role in soil environment and their strategic position at the base of the terrestrial food chain. In comparison with conventional bioassays using non-luminous fungal species, the measurement of bioluminescence as a toxicity endpoint is a faster, reproducible and more practical method. In this case, the influence of agar and nutrients on bioavailability is also lower, since exposure times are lower. The method described in this study is particularly advantageous due to the following features of *N. gardneri*: (i) rapid growth of mycelium, (ii) high sensitivity in agar medium, (iii) intense light emission, which enables testing in a wider concentration range, and (iv) consistency and predictability of bioluminescence profile in adjusted incubation conditions.

The proposed method adds another element to the scientist's repertoire to determine the toxicity of pollutants to terrestrial representatives: it can be considered an interesting complement to existing bioassay methods with other organisms. Considering that *N. gardneri* has not heretofore been used in any bioassays, this work is expected to contribute to expand tropical toxicological studies, in addition to serving as a basis for identifying new agricultural fungicides to target organisms, since some basidiomycetes are pathogenic. Thus, with the inclusion of *N. gardneri*, a representative of the Omphalotus lineage, all four lineages of bioluminescent fungi are now explored in the development of bioassays.

In conclusion, the results presented here as well as those found in the literature demonstrated that, regardless of the basidiomycete used (from Tropical or Temperate zones), the experimental condition of the bioassay (liquid or agar medium, 15 min or 24 h of exposure time), the mode of toxic action and the order of toxicity are similar.

## 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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Disclaimer – Authors confirm there are no conflicts of interest in the present study.

Data Accessibility – All data generated or analyzed during this study are included in this article.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2021.100044.

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