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Invited Review

Current Status of Research on Fungal Bioluminescence: Biochemistry and Prospects for Ecotoxicological Application†

Cassius V. Stevani*[1], Anderson G. Oliveira[2], Luiz F. Mendes[3], Fernanda F. Ventura[1,4], Hans E. Waldenmaier[1,3], Rodrigo P. Carvalho[1] and Tatiana A. Pereira[1]

[1]Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil
[2]Departamento de Genética e Evolução, Universidade Federal de São Carlos, Sorocaba, SP, Brazil
[3]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil
[4]Fundação Jorge Duprat Figueiredo de Segurança e Medicina do Trabalho, São Paulo, SP, Brazil

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ABSTRACT

Over the last half decade the study of fungal bioluminescence has regained momentum since the involvement of enzymes has been confirmed after over 40 years of controversy. Since then our laboratory has worked mainly on further characterizing the substances involved in fungal bioluminescence and its mechanism, as well as the development of an ecotoxicological bioluminescent assay with fungi. Previously, we proved the involvement of a NAD(P)H-dependent reductase and a membrane-bound luciferase in a two-step reaction triggered by addition of NAD(P)H and molecular oxygen to generate green light. The fungal luminescent system is also likely shared across all lineages of bioluminescent fungi based on cross-reaction studies. Moreover, fungal bioluminescence is inhibited by the mycelium exposure to toxicants. The change in light emission under optimal and controlled conditions has been used as endpoint in the development of toxicological bioassays. These bioassays are useful to better understand the interactions and effects of hazardous compounds to terrestrial species and to assist the assessment of soil contaminations by biotic or abiotic sources. In this work, we present an overview of the current state of the study of fungal luminescence and the application of bioluminescent fungi as versatile tool in ecotoxicology.

BIOLUMINESCENT FUNGI

The emission of light by living organisms has attracted the attention of mankind since the time of Aristotle (384–322 B.C.) and Pliny (23–79 A.D.) (1). The term bioluminescence was possibly first used by Harvey (2). It can be defined by the emission of visible and cold light by living organisms. There is a plethora of species described as bioluminescent (*e.g*. bacteria, fungi, dinoflagellates, marine and terrestrial animals). This phenomenon is distributed in nearly 700 genera of 16 major phyla, predominantly found in the oceans (3). The distribution of bioluminescent organisms in the phylogenetic tree of life is very intriguing, since there is no apparent relationship or rule for their distribution (2).

As of 2013, only 71 fungal species are known to be terrestrial and bioluminescent. They belong to four distinct evolutionary lineages in the order Agaricales: 52 from Mycenoid (Asia, Europe, the Americas, Africa, Caribbean, Australia and the Pacific Islands), 5 from Armillaria (one native of South/ Southeastern Asia and four of Europe/North America), 12 from Omphalotus (Asia, Europe, the Americas, Caribbean and Australia) and 2 from Lucentipes (*Gerronema viridilucens* and *Mycena lucentipes* found in Brazil) (4).

All bioluminescent fungi described to date, with the exception of some phytopathogens (*e.g*. *Armillaria mellea* and *Mycena citricolor*) are saprotrophic (5). They can be found in tropical and temperate areas, where the high humidity and warm climate favors their reproduction, growth and survival (5–8). All species are white-rot basidiomycete Agarics, which produce fruiting bodies (mushrooms) and whose mycelium secretes extracellular enzymes (*i.e*. laccases, lignin and manganese peroxidases) capable of degrading lignin (6,9,10).

The distribution of luminous tissues among fungal species is not uniform, in some species both mycelium and the whole basidiome are luminescent, while in others species only certain parts emit light. Fruiting bodies can also glow from only the stipe (stem), pileus (cap) or lamellae (gills) (3). Nevertheless, for all species the light is emitted as a continuous dim glow. One particularly interesting species is the fungus *Neonothopanus gardneri*, native to the states of Tocantins, Maranhão and Piauí in Brazil (4,11). The mycelium and large fruiting bodies of *N. gardneri* emit intense light (Fig. 1), both of which have been cultivated in our lab and are a staple of our research on the reaction mechanism.

MECHANISM OF LIGHT EMISSION

Raphaël Dubois demonstrated the first light emission from an in vitro experiment using a luciferin/luciferase system. Dubois used two extracts obtained from the light organs of the beetle *Pyrophorus noctilucus* (4,5,8,12). An extract was prepared with cold water, which resulted in a luminous suspension, and another in hot water, which eventually suppressed the luminosity. The glow of the cold extract decreased gradually until it disappeared. After cooling the hot extract, it was mixed with the cold extract and the light emission was restored. This was also observed by

*Corresponding author e-mail: stevani@iq.usp.br (Cassius V. Stevani)
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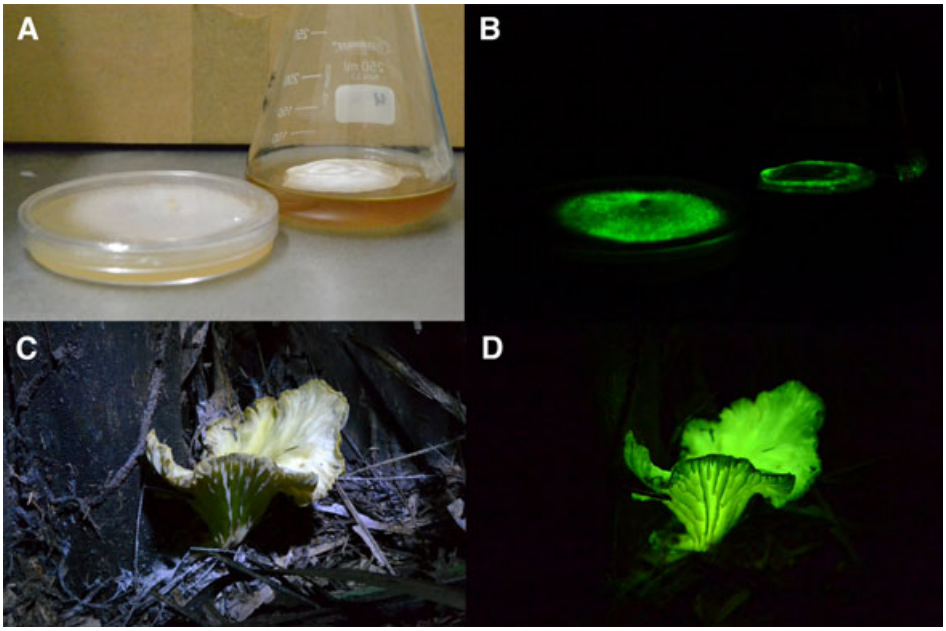

Figure 1. Bioluminescent mycelium cultures of the Brazilian fungus *Neonothopanus gardneri* in agar and liquid medium (A and B), and fruiting bodies growing on the base of a babaçu palm (*Attalea speciosa*) (C–D).

using hot and cold extracts from the clam *Pholas dactylus* (13). Based on these experiments, Dubois concluded that the cold extract comprised a heat labile enzyme factor, which was necessary for light emission. This fraction was designated by the French word luciférase. The hot extract contained a heat-stable factor, since all proteins were denatured by heat. This fraction was named luciférine. Therefore, it was determined that the light emission from the mixture of the two extracts was the result of a substrate/enzyme reaction.

Different organisms may possess different mechanistic pathways of light emission. As the light emission occurs only through a reaction that produces an excited state, it is reasonable to assume that all bioluminescent reactions are essentially chemiluminescence reactions (14). A bioluminescent reaction always involves the oxidation of a substrate (luciferin) by a luciferase or photoprotein (special case of luciferase where the substrate binds covalently with the protein). The formation of the emitter in the excited state is catalyzed by the luciferase, whose molecular structure and catalytic function are typically unique for different luminescent systems (2,15). In the jellyfish *Aequorea victoria* and some other luminescent marine organisms, the luciferin reacts first with oxygen inside the photoprotein (apoaequorin) to form a peroxide, which in turns binds covalently to the protein and yields the substrate/protein complex known as aequorin, which emits light upon the stimulus of a cationic flux (2). In all known systems, oxygen is required for the reaction to occur and results in the formation of the oxidized luciferin, oxyluciferin. Sometimes additional enzymes are required to convert the luciferin into a more reactive form that can then react with oxygen at the luciferase active site yielding light. This is the case for bacterial and fungal systems, where NADH/NADPH-dependent reductases are necessary (5,15) (Fig. 2).

The substrate of the bioluminescent reactions, the luciferin, is also unique to a given luminescent system with notable exceptions including coelenterazine and firefly luciferin (3,4). Coelenterazine is the luciferin of a few marine bioluminescent organisms from six phyla [*e.g.* Sarcomastigophora (protozoa),

Bacterial bioluminescence

$$FMN + NAD(P)H + H^+ \xrightarrow{reductase} FMNH_2 + NAD(P)^+$$

$$FMNH_2 + RCHO + O_2 \xrightarrow{luciferase} FMN + RCO_2H + H_2O + h\nu$$

FMN: oxidized flavin mononucleotide (luciferin), $FMNH_2$: reduced FMN
RCHO: fatty aldehyde, RCO_2H: fatty acid

Fungal bioluminescence
- Airth & Foerster proposal

$$L + NAD(P)H + H^+ \xrightarrow{reductase} LH_2 + NAD(P)^+$$

$$LH_2 + O_2 \xrightarrow{luciferase} LO + H_2O + h\nu$$

L: luciferin, LH_2: reduced luciferin, LO: oxyluciferin

Figure 2. Postulated mechanisms involved in the bioluminescence of bacteria and fungi.

Cnidaria, Ctenophora, Mollusca, Arthropoda, and Chordata (pisces)] (4). The firefly luciferin occurs in all families of characterized luminescent beetles (*i.e.* Lampiridae, Elateridae and Phengodidae). Usually, both luciferin and luciferase are used as generic terms to refer to any substrate and enzyme involved in a bioluminescent system. Additionally, bioluminescence should not be confused with ultraweak chemiluminescence, which is a process connected to oxidative stress and the production of reactive oxygen and carbonyl species originating from singlet oxygen, triplet states, peroxynitrite, lipid peroxidation, and reactions between heme proteins and peroxides (2,16). This review article is centered on fungal bioluminescence defined as a chemical reaction inside a basidiomycete that yields green light with maximum intensity in the range 520–530 nm (5).

In 1959, Airth and McElroy successfully observed light from an in vitro reaction using hot and cold extracts from bioluminescent fungi (17). The addition of either NADH or NADPH [NAD(P)H for short] to the cold and hot extracts was required to trigger the emission of light. Airth and McElroy also described that, after ultracentrifugation, the proteinaceous cold extract could be

further separated in two fractions, a soluble (supernatant) and an insoluble (pellet) fraction. Each fraction contained an essential enzyme for the light emission reaction, suggesting a two-step consecutive enzymatic pathway. The first step involves a dark reaction between NAD(P)H, the luciferin, and a soluble enzyme in the supernatant, followed by the reaction of the reduced substrate luciferin with molecular oxygen catalyzed by the enzyme present in the resuspended pellet that results in light emission (Fig. 2).

There was some controversy in the past about the involvement of enzymes in fungal system (2,5,18). In 2009, Oliveira and Stevani successfully verified Airth and Foerster's work using hot and cold extracts from luminous fungi, demonstrating that fungal bioluminescence indeed depends on a NAD(P)H-dependent reductase and a luciferase (17–20). The fungal luciferin reacts initially with a NAD(P)H-dependent reductase, and the reduced luciferin intermediate is oxidized enzymatically by the luciferase, resulting in light, with an overall reaction similar to bacterial bioluminescence. Oliveira and Stevani were also able to record the emission spectrum of the reaction in vitro, which proved to match the in vivo one, providing additional evidence that both process are related (18). In 2012, it was demonstrated that cross-reactions of hot and cold extracts from mycelium of different luminescent species (cultivated on agar and liquid media containing sugarcane molasses and yeast extract) representing the four lineages (Armillaria, Lucentipes, Mycenoid and Omphalotus) of bioluminescent fungi were compatible with each other. Extracts from non-luminescent species did not result in light emission when cross-reacted (4). These results strongly suggest that all four lineages of luminescent fungi share similar or compatible types of luciferin/luciferase and that the biochemical mechanism leading to light production could be of only one kind in the Fungal Kingdom.

The investigation of fungal bioluminescence would not be complete without examining its ecological function and its evolution, which is particularly interesting given that so few species (only 71) are luminescent and are widely distributed in the Agaricales (with 9,000 species), yet all share comparable mechanisms leading to light production. Even more puzzling, unlike most other bioluminescent organisms, fungi have no recognized means to detect their own light or the light of others. Therefore, the significance of fungal bioluminescence could likely involve interactions with other organisms. Sivinski performed an investigation of the interaction of arthropods and luminous fungi in the early 1980s (21). Glass vials coated with adhesive were used to cover luminescent mushrooms and mycelium, and these traps were then placed in the locations where luminous mushrooms grow. Arthropods such as Collembola and Diptera were observed more frequently on luminescent glue traps than controls. In his work, Sivinski presented several hypotheses for the ecological significance of fungal bioluminescence: (1) to attract spore dispersers, carnivores of fungivores, fungivores of other fungal competitors, or the attraction of fertilizers, (2) to repeal negatively phototrophic fungivores, (3) and as an aposematic signal. Investigations of the ecological significance of fungal bioluminescence have not been performed since Sivinski's in the 1980s. Currently we are performing similar experiments in the field to address the ecological function of fungal bioluminescence in two distinct geographic regions of Brazil based on previous work reported by Sivinski.

Our group has been developing different thematic works, which address the mechanism, ecological function and the application of fungal bioluminescence in a toxicological assay. We are working on the chemical characterization of an active compound, the putative fungal luciferin, which is capable of emitting green light upon addition of NAD(P)H, oxygen, reductase and luciferase (22,23). We are also interested to isolate and characterize the reductase, the luciferase and their corresponding genes through conventional chromatographic, electrophoretic, and mass spectrometry methods. Along with this we are also conducting a bioinformatical investigation of the recently published draft genome of *Omphalotus olearius* (24) and the transcriptome data of *N. gardneri* with the aim to identify the genes coding for both enzymes. Attempts to purify the enzymes and the luciferin in the early 1960s were unsuccessful (20,25). In the case of bioluminescent bacteria, with an overall reaction mechanism similar to fungi, the enzyme purification was successfully performed in the 1970s (26). Although bacterial bioluminescence resembles fungal regarding the involvement of a reductase and a luciferase, these enzymes are not the same in both organisms, but belong to the same functional class. The bacterial bioluminescence proteins are soluble and can be found in the bacterial cytosol, but fungal luciferase is most probably associated with a membrane. Moreover, neither FMN nor any flavin derivative/moiety is active as luciferin in fungi (8). There is also no need of a fatty aldehyde in the fungal system. Nevertheless, the bacterial system can be used as a useful model for the characterization of the fungal enzymes. As mentioned, one significant difference between the fungal and bacterial systems is that the fungal luciferase is associated with membrane-rich fractions (18–20). The use of various surfactants has been shown independently to increase luminescence of homogenate extracts of *Mycena chlorophos* (7), suggesting that a component of the bioluminescent pathway is indeed membrane-bound. Understanding the biochemistry of light production in fungus will help us understand how to interpret changes in the bioluminescence upon exposure to variable environmental conditions, and contaminants. Change of light production upon exposure to contamination, and presumably stress, makes luminous fungi attractive for application as ecotoxicological tools.

ECOTOXICOLOGICAL APPLICATION

Relevance of fungi in soil ecosystem

There are at least 1.5 million species of fungi distributed worldwide, among which only about 100 000 were scientifically identified and cataloged (4,27). Fungi are well known to be a large and diverse group that play essential role in economic activities, such as the manufacturing of foods and antibiotics, synthesis of organic compounds, enzymes and vitamins (28).

Basidiomycete fungi are decomposer organisms involved in the recycling of nutrients from decaying matter to upper trophic levels in the soil food chain (29,30). They reproduce either sexually by production of basidiospores in rod-shaped structures called basidia, originating fruiting bodies (mushrooms), or asexually by fragmentation and asexual sporulation. The spores germinate yielding the mycelium: a group of tubular and cylindrical structures called hyphae, used for substrate fixation, reproduction and fungal nutrition (30,31). Besides the lignin-degrading activity, the main ecological significance of basidiomycete fungi is their symbiotic relationship with other living organisms coexisting in the soil environment, especially plants. They are able to

solubilize and also immobilize metals and inorganic ions present in soil. The nutrients can be used by other life forms from all trophic levels, contributing to their survival and, thereby, maintaining ecosystem health (29,30,32). On the other hand, metal toxicants in the environment can be immobilized or transformed to a lesser toxic form and thus preventing the contact of harmful substances with soil organisms (33–35).

Toxicity of organic and inorganic substances

Living organisms are subject to a wide variety of toxic organic and inorganic compounds in the environment, from both biotic and abiotic sources (36). These toxicants can affect fungal sporulation and their metabolism causing alterations in reproduction, growth, light emission (in the case of bioluminescent species), enzymes expression and activity. Environmental characteristics also have influence on the toxicity, depending on physicochemical properties of soil, which can affect the availability of toxic compounds (28,37–40).

Interactions of toxicants with fungi are widely assorted, especially because of the great amount of soil compounds in several speciation forms, in addition to the large variety and complexity of organisms and their metabolic processes. Generally, light emission decreases as a response to the exposure to a toxicant, but in some cases it can also increase (41). This could be partially explained by hormesis, which is known to cause a positive response from organisms exposed to certain doses of hazardous substances (42,43).

Some toxic organic and inorganic compounds, such as phenols and metal cations, cause depolarization of cell membranes, interfering with the cellular electrochemical gradient and thus affecting nutrients uptake, ions exchange with the outer environment and uncoupling the mitochondrial oxidative phosphorylation (28,35,44,45). Moreover, toxic agents can also cause several other deleterious effects by intra and extracellular enzymatic inhibition and denaturation, replacement of essential ions from biomolecules and blockage of important biological functional groups.

Some toxicants interact with organisms to form reactive oxygen species (ROS), which have a high reactivity with biomolecules. They can promote lipid peroxidation and damage to cellular membranes (46). Phenols, for example, are initially oxidized by enzymes in a one-electron redox process, producing highly reactive phenoxy radicals and ROS (*e.g.* hydrogen peroxide) that depolarize membranes of cell walls, mitochondria and nucleus (47). Additionally, some metals as Cd(II) and Hg(II) inactivate enzymes (*i.e.* catalase, superoxide dismutase), whose function is to protect the organism against the deleterious action of ROS. The inhibition of such enzymes can increase indirectly the concentration of ROS as byproduct of normal respiration (28).

Although the toxicity mechanism and its specific influence on the fungal bioluminescence reaction are still not known in detail, the uncoupling of oxidative phosphorylation and the depolarization of mitochondrial membranes by toxic compounds can be pointed out as possible pathways (28,35,44,45). The decrease in light emission might be a result of those effects, which could both restrain the electron transport system through the mitochondrial membrane and affect ATP synthesis by oxidative phosphorylation. This could indirectly affect the NADH availability to the reductase involved in the bioluminescent reaction, as this cofactor would be preferably used to produce ATP. Moreover, the biosynthesis of NADP+ occurs with the phosphorylation of NAD+ by NAD+ kinase in the presence of ATP (48). Therefore, the production of NADPH (that can also be used in fungal bioluminescence) by reduction of NADP+ could be also affected, as the available NAD+ would be used preferentially to produce more NADH and prioritize ATP biosynthesis.

Fungal toxicological bioassays

Basidiomycetes have been considered in the development of toxicological bioassays due to their role as lignin decomposers, their relationship with plants, the ecological trophic position in soil food chain and its ability to cycle essential nutrients (28,32,49,50). Fungal toxicological bioassays are useful to evaluate possible changes in growth, biomass, reproduction and cellular viability, and are important to assist the prevention and the assessment of environmental contamination caused by potentially toxic chemicals present in environmental matrices (51–55). Moreover, fungal bioassays can help the development of more efficient fungicides to control crop pathogens and, thereby, increase the agricultural productivity (28).

Toxicological assays using eukaryotic bioluminescent fungi are complementary to the commonly used assays with luminous bacteria (Microtox®, for example) that, unlike fungi, are mainly aquatic organisms. Both types of microorganisms occupy the first trophic level of each food chain and have differences in natural habitats, organism defense machinery and toxicant speciation/bioavailability in each specific environment as well. Hence, it is reasonable to assume that fungi are a more appropriate class of organisms to perform toxicological studies with soil samples as they represent more accurately the response of terrestrial species to soil toxicants. Bacteria, on the other hand, should be better representatives for toxicological assessment of aqueous samples (32,50).

Non-luminescent fungal bioassays with basidiomycetes (hereafter called conventional fungal assays) are based on the measurement of biomass and/or radial growth rates, in the presence and absence of toxic agents added to the medium in a wide range of concentrations (51,52,55). Mycelium cultures are maintained in climatic chambers on agar medium with previously determined optimal pH, temperature and nutrients at high humidity (49).

Conventional fungal assays demonstrate some inconveniences such as long-term observation (up to 30 d). They are sometimes difficult to interpret as growth rates are often assessed by expansion in the horizontal plane (two dimensions or surface area), while the fungal mycelium also grows upward perpendicular to the plane of measurement. Finally, the agar medium and its constituents can change the bioavailability of toxicants to the fungus, which could explain why conventional fungal assays normally exhibit higher median effective concentration (EC_{50}) values than those carried out in liquid media (32). Conventional assays using the species *Thelephora terrestris* showed that the EC_{50} values for Cu(II) and Zn(II) were *ca.* 10 mgL^{-1} in liquid medium (56), but 100–500 and 1,000 mgL^{-1}, respectively on agar (57).

Bioassays with bioluminescent fungi are less commonly used than either bioluminescent or non-bioluminescent organisms (*e.g.* bacteria, daphnids and algae) (6,9,58–60). The procedure consists in the inoculation of either the agar medium or liquid media with the glowing mycelium (32,50). In this case, however, the endpoint chosen is light emission instead of the radial growth

rates. Several bioassays with bioluminescent basidiomycetes have been developed using the species *Armillaria mellea*, *Panellus stipticus*, *Mycena citricolor*, *Gerronema viridilucens* and *Omphalotus olearius*. These species were exposed to phenols, quinones and metal cations (32,49,50,53,54,61–63). In general, bioassays were carried out in solid or liquid media at 15–25°C, pH 3–6, 80%–90% humidity and under dark conditions inside a climatic chamber during an incubation period between 7 and 10 days.

Carbon sources include sugar cane molasses, monosaccharides or potato dextrose. Yeast extract, malt extract, ammonium or L-asparagine is commonly used as nitrogen sources. The exposure time of the mycelium to the toxicant varies from 5 min to 24 h (Table 1). It has been shown in a study on the effect of culture conditions on growth and bioluminescence of *Panellus stipticus* that complex carbon sources do not promptly release monosaccharides (mainly glucose) to the medium and thus are less favorable to be used in cultures, leading to lower growth rates and lower light emission (61). Carbon sources commonly available in wood-decaying environments, such as pectin, lactose, maltose and glucose, on the other hand, resulted in higher bioluminescence, as a consequence of the rapid absorption of the nutrients by the mycelium.

Initial reports on the development of fungal-based luminescent assays were conducted in stirred liquid medium with the globular mycelium (50,53,54). The authors evaluated the toxicity of phenols, metal cations, and also were able to perform some tests with sewage sludge (53).

Later on, a fungal-based luminescent assay was proposed on agar plates using the fungus *Gerronema viridilucens* (32). The procedure involves the cultivation of the mycelium in 100 mm Petri dishes (used to continue the cultures) for 20 days in optimized conditions inside a climatic chamber, followed by (1) the inoculation of 35 mm dishes (35 to 40 dishes and maintenance for 10 days in the same optimal conditions), (2) the measurement of the integral of initial bioluminescence from each 35 mm dishes ($BL_{initial}$), (3) the addition (under gentle swirling of the dish) of 500 μL of either the aqueous control [0.050% (w:v) Triton X-100 at pH 5.5–6.0 non-buffered] or the toxicant solution at increasing concentrations, followed by a 24 h exposure time in the climatic chamber, and (4) the measurement of the final bioluminescence (BL_{final}). The inhibition of bioluminescence (BL_{inhib}) can be calculated by the expression: $BL_{inhib} = 1 - BL_{final}/BL_{initial}$ (Fig. 3).

One of the advantages of bioluminescent assays on agar over the conventional ones is that each dish acts as its self-control. This is important to decrease experimental errors associated with different mycelium growing in different plates, which could lead to less reliable results (32). The bioluminescence signal is more accurate for data acquisition and verification of fungal organism status as it reflects the condition of all mycelium layers, and is a precocious endpoint for fungal injury. As previously reported, results based on area measurement (radial growth rate) do not consider the mycelium density and thus can underestimate the mycelium growth (49,61).

It is noteworthy to mention that some experimental parameters can strongly affect EC_{50} values obtained from bioassays. Among them it is possible to include: the medium composition (*i.e.* liquid or agar, pH, temperature and nutrients), exposure time to toxicants and the intrinsic variability of species. The EC_{50} values obtained from a toxicological bioluminescent assay using the fungus *A. mellea* in liquid medium for Cu(II) and Zn(II) were 1.3

Table 1. Optimal conditions for growth and bioluminescence (BL) emission available in the literature for luminous fungus.

Species	*t* (°C)		pH		Nutrients	Study purpose	References
	Growth	BL	Growth	BL			
G. viridilucens	25		4.2	5.5–6.0	Yeast extract, cane molasses in non-buffered agar media	Effects of culture conditions on BL and bioassay for metals	(32,49)
A. mellea	22		—	3.0–4.0	YM broth and PD broth in buffered media	Bioassay for metals and phenols	(50)
M. citricolor				4.0–5.0			
A. mellea	22		5		YM broth and YM agar in buffered media	Biosensor to heavy metals in sewage sludge on forest soils	(53)
A. mellea	22		5		ME agar and ME broth in buffered media	Biosensor to heavy metals in sewage sludge on forest soils	(54)
P. stipticus	28	22	3.8		Ammonium or asparagine and glucose, maltose, lactose, trehalose, cellobiose or pectin in agar buffered media	Effects of culture conditions on mycelial growth and BL	(61)
A. mellea, A. gallica *L. japonicus* *A. borealis*	room temperature		—		Agarized Saburo	Effect of toxic inorganic and organic compounds on BL	(41)
A. mellea	22		3.5		Oxoid malt extract in buffered media	Effects of culture conditions on growth and BL	(62)
M. citricolor			4.0	5.0–6.0			
P. stipticus			3.5–4.0	3.5			
O. olearius	22	15	3.5	3.0			
P. stipticus	22		—	—	White bread crumb in agar non-buffered media	BL as an endogenous reporter system to evaluate biological control agents	(63)
O. olearius							

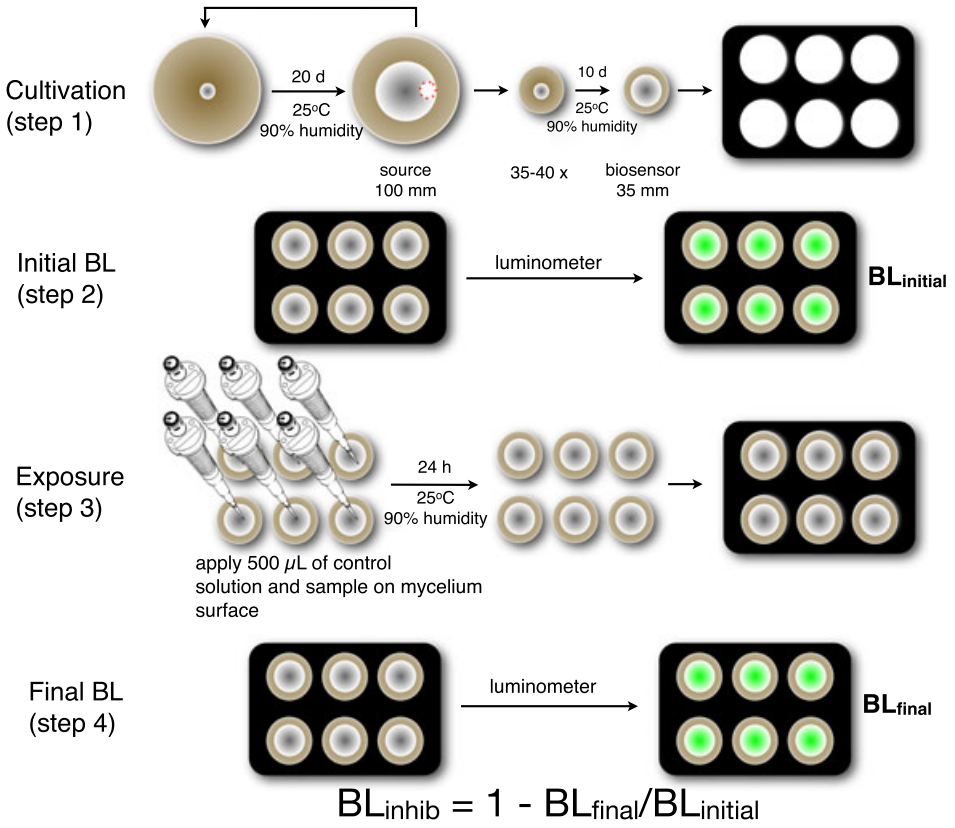

Figure 3. Step-by-step description of the fungal-based bioluminescent assay proposed by Mendes and Stevani (32).

and 490 μmolL^{-1}, respectively. On the other hand, the EC_{50} values obtained with the fungus *M. citricolor* were *ca.* 30- and 3-fold higher for the same metal cations (50). The values obtained with these metal cations, but using an agar-based bioassay and the bioluminescent fungus *G. viridilucens* were 5.7 and 48 mM (32). Although the species and experimental conditions are different, changes in EC_{50} values are probably related to the high tolerance of *G. viridilucens* to the metal cations due to molecular defense mechanisms such as: (1) extracellular immobilization by precipitation, complexation and/or crystallization, (2) biochemical transformation of toxic metals (by oxidation, reduction and methylation, for example), (3) biosorption by macromolecules in the cell walls (*e.g.* melanin and chitin), (4) flow and counter flow of ions through cellular membranes and (5) vacuolar and intracellular immobilization by compartmentalization (28,32,35).

Although the assays performed in liquid medium have higher repeatability and the light emission remains constant longer than in agar medium - as the interference of humidity variation during mycelium cultivation is prevented – the liquid medium does not reproduce the mycelial growth and differentiation in soil (32,33,64). Thus, the bioavailability of toxic compounds could be possibly less representative in liquid medium compared to bioluminescent assays in agar. A comparison between bioluminescent and non-bioluminescent fungal toxicological assays is summarized in Table 2.

In general, toxicological studies using bioassays are time-consuming and labor-intensive, demanding trained personal to maintain the organisms and to perform the experiments. Hence, it is very interesting to investigate and generate linear free energy relationships that can reasonably predict the toxicity of different classes of substances to a specific target organism. Some prediction models rely on the Quantitative Structure-Activity Relationships (QSARs, for organic compounds) or Quantitative Ion Character-Activity Relationships (QICARs, for inorganic species) to estimate the toxicity of non-tested compounds to the target organism based on the values previously obtained (65). Experimentally determined EC_{50} values are plotted against physical-chemical properties of organic and inorganic compounds and the relationships with the highest correlation coefficients are chosen. The compound pK_a, octanol-water constant, ionic radius, atomic number, electronegativity, covalent index, first hydrolysis constant, softness index, ion charge, ionization potential, electrochemical potential, and the cation polarizing power are

Table 2. Comparison between conventional and bioluminescent-based (BL) toxicological assays using basidiomycete fungi.

Parameter	Conventional (agar)	BL (agar)	BL (liquid medium)
Measurement	Diameter and biomass	Light emission	Light emission
Endpoint	Growth inhibition	BL inhibition	BL inhibition
Exposure time	7–30 days	24 h	60 min
Toxicant application	Inside agar	Agar surface	In solution
Controls	No self-control	Self-controlled	Self-controlled
EC_{50} values	µM–mM	mM	µM–mM
References	(33,56,57)	(32)	(50,53,54)

some examples of parameters that can be used in QSAR or QICAR approaches (65).

In the case of fungi, a QICAR approach has been used to evaluate and predict the toxicity of eleven mono- and divalent metal cations to the bioluminescent species *G. viridilucens* (66). Nineteen different regressions based on univariate, two- and three-variable models were generated. The covalent index ($X_m^2 r$), which indicates the binding tendency between the metal cation and soft ligands (*e.g.* the preference to bind to sulfur donor atoms) demonstrated to be the most adequate parameter for the prediction of fungal metal toxicity [log EC_{50} = 4.243 (±0.243) − 1.268 (± 0.125)$X_m^2 r$, R^2 = 0.9113], indicating that covalent bonding - most probably forming stable nonlabile complexes with biomolecules, especially sulfur-containing ones - is an important factor of metal inherent toxicity to fungi (66).

Fungal-based bioluminescent assays using white-rot basidiomycete fungi could represent an effective additional option to the already employed conventional bioassays for those who aim to obtain further and reliable information about the toxicity of pure substances, environmental samples and agriculture commercial fungicides. Moreover, despite the importance of the identification and quantification of toxicants in environmental matrices using analytical equipment, the concentration itself does not reflect the bioavailability of contaminants in nature or their potential damage to living organisms, which could be caused by a synergistic effect of two or more substances (67). Therefore, bioassays using bioluminescent fungi are needed as a complementary test not only to the already implemented and well established bioassays using aquatic and terrestrial species, but also to traditional analytical techniques.

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AUTHOR BIOGRAPHIES

Cassius V. Stevani was born in São Paulo, SP. He received his BSc in Chemistry in 1992 from the Chemistry Institute of University of São Paulo (IQ-USP). He completed his PhD in Organic Chemistry in 1997 under supervision of Prof. J. W. Baader and was a post-doctoral fellow working with Prof. E. J. H. Bechara (1998–2000) both at IQ-USP. Currently, he lectures in Organic and Environmental Organic Chemistry as Associate Professor at IQ-USP, where his group studies the mechanism and environmental application of bioluminescent fungi.

Anderson G. Oliveira was born in Agudos, SP. He received his BSc in Chemistry in 2004 from the Universidade Federal de Santa Catarina in Florianópolis (UFSC). In 2005 he moved to São Paulo to study the mechanism of fungi bioluminescence under the supervision of Prof. Cassius Vinicius Stevani at IQ-USP. He completed his PhD in Organic Chemistry in 2010. He was a post-doctoral fellow working with Prof. Vadim Viviani at Federal University of São Carlos (2010-2013). Recently, He was hired as Assistant Professor at Federal University of São Paulo (UNIFESP) in São José dos Campos.

Luiz F. Mendes was born in Carapicuíba, SP. He is BSc in Chemistry by UNIFIEO, Osasco and PhD in Chemistry (2010) by IQ-USP under supervision of Prof. Cassius V. Stevan. Since, 2010 he is a post-doctoral fellow working with Prof. P. Colepicolo at IQ-USP Biochemistry Department, studying fungal and algae toxicological assays.

Fernanda F. Ventura received her BSc in Chemistry in 2010 from Faculdades Oswaldo Cruz in São Paulo. In 2011 she began her MSc under supervision of Prof. Cassius V. Stevani at IQ-USP to develop a toxicological assay using bioluminescent fungi. Currently, she works at Fundacentro as Chemist.

Hans E. Waldenmaier was born in White Plains, New York. He received his BSc in Biochemistry in 2009 from Clemson University in South Carolina. In 2011 he received his MSc in Botany from Miami University in Oxford, Ohio. He began his PhD work in Biochemistry under supervision of Prof. Cassius V. Stevani at IQ-USP in order to study the molecular biology and ecology of fungal bioluminescence.

Rodrigo P. Carvalho was born in São José dos Campos, SP. He received his BSc in Chemistry in 2012 from IQ-USP. In the same year, he started his PhD under supervision of Prof. Cassius V. Stevani to study the mechanism of fungi bioluminescence.

Tatiana A. Pereira was born in Nova Iguaçú, RJ. She received her BSc in Environmental Chemistry in 2010 and her MSc in 2012 from IQ-USP. Now she is pursuing her PhD studying the enzymes involved in fungal bioluminescence under the supervision of Prof. Cassius V. Stevani.

REFERENCES

1. Harvey, E. N. (1952) *Bioluminescence*. Academic Press, New York, NY.
2. Shimomura, O. (2006) *Bioluminescence: Chemical Principles and Methods*. World Scientific Publishing Co. Pte. Lt, Singapore.
3. Waldenmaier, H. E., A. G. Oliveira and C. V. Stevani (2012) Thoughts on the diversity of convergent evolution of bioluminescence on earth. *Int. J. Astrobiol.* **11**, 335–343.
4. Oliveira, A. G., D. E. Desjardin, B. A. Perry and C. V. Stevani (2012) Evidence that a single bioluminescent system is shared by all known bioluminescent fungal lineages. *Photochem. Photobiol. Sci.* **11**, 848–852.
5. Desjardin, D. E., A. G. Oliveira and C. V. Stevani (2008) Fungi bioluminescence revisited. *Photochem. Photobiol. Sci.* **7**, 170–182.
6. Deheyn, D. D. and M. I. Latz (2007) Bioluminescence characteristics of a tropical terrestrial fungus (Basidiomycetes). *Luminescence* **22**, 462–467.
7. Mori, K., S. Kojima, S. Maki, T. Hirano and H. Niwa (2011) Bioluminescence characteristics of the fruiting body of *Mycena chlorophos*. *Luminescence* **26**, 604–610.
8. Oliveira, A. G., R. P. Carvalho, H. E. Waldenmaier and C. V. Stevani (2013) Bioluminescência de fungos: distribuição, função e mecanismo de emissão de luz. *Quím. Nova* **36**, 314–319.
9. Hastings, J. W. (2004) *The Desk Encyclopedia of Microbiology*. Elsevier Academic Press, San Diego, CA.
10. Leonowicz, A., A. Matuszewska, J. Luterek, D. Ziegenhagen, M. Wojtaś-Wasilewska, N.-S. Cho, M. Hofrichter and J. Rogalski (1999) Biodegradation of lignin by white rot fungi. *Fungal Genet. Biol.* **27**, 175–185.
11. Capelari, M., D. E. Desjardin, B. A. Perry, T. Asai and C. V. Stevani (2011) *Neonothopanus gardneri*: A new combination for a bioluminescent agaric from Brazil. *Mycologia* **6**, 1433–1440.
12. Dubois, R. (1885) Note sur la physiologic des pyrophores. *C. R. Séanc. Soc. Biol. Fr.* **37**, 559–562.
13. Dubois, R. (1887) Note sur la fonction photogénique chez le *Pholas dactylus*. *C. R. Séanc. Soc. Biol. Fr.* **39**, 3.
14. Campbell, A. K. (1988) *Chemiluminescence: Principles and Applications in Biology and Medicine*. VCH, Chichester.
15. Wilson, T. and J. W. Hastings (1998) Bioluminescence. *Ann. Rev. Cell Biol.* **14**, 197–230.
16. Halliwell, B. and J. M. C. Gutterridge (2007) *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford.
17. Airth, R. L. and W. D. McElroy (1959) Light emission from extract of luminous fungi. *J. Bacteriol.* **77**, 249–250.
18. Oliveira, A. G. and C. V. Stevani (2009) The enzymatic nature of fungal bioluminescence. *Photochem. Photobiol. Sci.* **8**, 1416–1421.
19. Airth, R. L. and G. E. Foerster (1962) The isolation of catalytic components required for cell-free fungal bioluminescence. *Arch. Biochem. Biophys.* **97**, 567–573.
20. Airth, R. L., G. E. Foerster and Q. P. Behrens (1966) The luminous fungi. In *Bioluminescence in Progress*. (Edited by F. H. Johnson and Y. Haneda), pp. 203–233. Princeton University Press, NJ.
21. Sivinski, J. (1981) Arthropods attracted to luminous fungi. *Psyche* **88**, 383–390.
22. Oliveira, A. G., R. P. Carvalho and C. V. Stevani (2012) On the purification of the fungal luciferin. *Luminescence* **27**, 150.
23. Oliveira, A. G., R. P. Carvalho, H. E. Waldenmaier, V. R. Viviani and C. V. Stevani (2012) On the purification of the NAD(P)H-dependent reductase involved in fungal bioluminescence. *Luminescence* **27**, 151.
24. Wawrzyn, G. T., M. B. Quin, S. Choudhary, F. Lopez-Gallego and C. Schmidt-Dannert (2012) Draft genome of *Omphalotus olearius* provides a predictive framework for sesquiterpenoid natural product biosynthesis in Basidiomycota. *Chem. Biol.* **19**, 772–783.
25. Airth, R. L. and G. E. Foerster (1964) Enzymes associated with bioluminescence in *Panus stypticus* luminescens and *Panus stypticus* nonluminescens. *J. Bacteriol.* **88**, 1372–1379.
26. Jablonski, E. and M. Deluca (1977) Purification and properties of the NADPH specific FMN oxidoreductases from *Beneckea harveyi*. *Biochemistry* **16**, 2932–2936.
27. Hawksworth, D. L. (2012) Global species numbers of fungi: Are tropical studies and molecular approaches contributing to a more robust estimate? *Biodivers. Conserv.* **21**, 2425–2433.
28. Gadd, G. M. (1993) Interactions of fungi with toxic metals. *New Phytol.* **124**, 25–60.
29. Kabir, Z., I. P. O'Halloran and C. Hamel (1997) Overwinter survival of arbuscular mycorrhizal hyphae is favored by attachment to roots but diminished by disturbance. *Mycorrhiza* **7**, 197–200.
30. Wessels, J. G. H. (1992) Gene expression during fruiting in Schizophyllum commune. *Mycol. Res.* **96**, 609–620.
31. Griffin, D. H. (1994) *Fungal Physiology*. Wiley Science, New York, NY.
32. Mendes, L. F. and C. V. Stevani (2010) Evaluation of metal toxicity by a modified method based on the fungus *Gerronema viridilucens* bioluminescence in agar medium. *Environ. Toxicol. Chem.* **29**, 320–326.
33. Hartley, J., J. G. Cairney and A. Meharg (1997) Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? *Plant Soil* **189**, 303–319.
34. Lasat, M. M. (2002) Phytoextraction of toxic metals. *J. Environ. Qual.* **31**, 109–120.
35. Meharg, A. A. (2003) The mechanistic basis of interactions between mycorrhizal associations and toxic metal cations. *Mycol. Res.* **107**, 1253–1265.
36. Vasconcellos, P. C., D. Z. Souza, O. Sanchez-Ccoyllo, J. O. V. Bustillos, H. Lee, F. C. Santos, K. H. Nascimento, M. P. Araújo, K. Saarnio, K. Teinilä and R. Hillamo (2010) Determination of anthropogenic and biogenic compounds on atmospheric aerosol collected in urban, biomass burning and forest areas in São Paulo. *Brazil. Sci. Total Environ.* **408**, 5836–5844.
37. Anahid, S., S. Yachmaei and Z. Ghobadinejad (2011) Heavy metal tolerance of fungi. *Sci. Iran* **18**, 508–508.
38. Babich, H., C. Gamba-Vitalo and G. Stotzky (1982) Comparative toxicity of nickel to mycelial proliferation and spore formation of selected fungi. *Arch. Environm. Contam. Toxicol.* **11**, 465–468.
39. Baldrian, P. (2003) Interactions of heavy metals with white-rot fungi. *Enzyme Microb. Techn.* **32**, 78–91.

40. Cairney, J. W. G. and A. A. Meharg (1999) Influences of anthropogenic pollution on mycorrhizal fungal communities. *Environ. Pollut.* **106**, 169–182.
41. Vydryakova, G. A., A. A. Gusev and S. E. Medvedeva (2011) Effect of organic and inorganic toxic compounds on luminescence of luminous fungi. *Appl. Biochem. Michobiol.* **47**, 293–297.
42. Kending, E. L., H. H. Le and S. M. Belcher (2010) Defining hormesis: Evaluation of a complex concentration response phenomenon. *Int. J. Toxicol.* **29**, 235–246.
43. Calabrese, E. J. (2008) Hormesis: Why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem.* **27**, 1451–1474.
44. Belgers, J. D. M., G. G. Aalderink and P. J. Van den Brink (2009) Effects of four fungicides on nine non-target submersed macrophytes. *Ecotoxicol. Environ. Saf.* **72**, 579–584.
45. Laiho, R. (2006) Decomposition in peatlands: Reconciling seemingly contrasting results on the impacts of lowered water levels. *Soil Biol. Biochem.* **38**, 2011–2024.
46. Halliwell, B. and S. Chirico (1993) Lipid peroxidation: Its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* **57**, 715S–752S.
47. Michałowicz, J. and W. Duda (2006) Phenols – Sources and toxicity. *Polish J. Environ. Stud.* **16**, 347–362.
48. Ying, W. (2008) NAD^+/NADH and $NADP^+$/NADPH in cellular functions and cell death: Regulation and biological consequences. *Antioxid. Redox Sign.* **10**, 179–206.
49. Mendes, L. F., E. L. Bastos, D. E. Desjardin and C. V. Stevani (2008) Influence of culture conditions on mycelial growth and bioluminescence of *Gerronema viridilucens*. *FEMS Microbiol. Lett.* **282**, 132–139.
50. Weitz, H. J., C. D. Campbell and K. Killham (2002) Development of a novel, bioluminescence-based, fungal bioassay for toxicity testing. *Environ. Microbiol.* **4**, 422–429.
51. Colpaert, J. V. and J. A. Assche (1987) Heavy metal tolerance in some ectomycorrhizal fungi. *Dunct. Ecol.* **1**, 415–421.
52. Gadd, G. M., J. L. Mowll, C. White and P. J. Newby (1986) Methods for assessment of heavy metal toxicity towards fungi and yeasts. *Toxic Assess.* **1**, 169–185.
53. Horswell, J., H. Weitz, H. Percival and T. Speir (2006) Impact of heavy metal amended sewage sludge on forest soils as assessed by bacterial and fungal biosensors. *Biol. Fert. Soils* **42**, 569–576.
54. Paton, G. I., E. Viventsova, J. Kumpene, M. J. Wilson, H. J. Weitz and J. J. C. Dawson (2006) An ecotoxicity assessment of contaminated forest soils from the Kola Peninsula. *Sci. Total Environ.* **355**, 106–117.
55. Ray, P., R. Tiwari, U. Reddy and A. Adholeya (2005) Detecting the heavy metal tolerance level in ectomycorrhizal fungi in vitro. *World J. Microb. Biot.* **21**, 309–315.
56. Tam, P. C. F. (1995) Heavy metal tolerance by ectomycorrhial fungi and metal amelioration by *Pisolithus tinctorius*. *Mycorrhiza* **5**, 181–187.
57. Jones, D. and A. Muehlchen (1994) Effects of the potentially toxic metals, aluminium, zinc and copper on ectomycorrhizal fungi. *J. Environ. Sci. Health A* **29**, 949–966.
58. Kramer, K. J. M., R. G. Jak, B. van Hattum, R. N. Hooftman and J. J. G. Zwolsman (2004) Copper toxicity in relation to surface water-dissolved organic matter: Biological effects to *Daphnia magna*. *Environ. Toxicol. Chem.* **23**, 2971–2980.
59. McCloskey, J. T., M. C. Newman and S. B. Clark (1996) Predicting the relative toxicity of metal ions using ion characteristics: Microtox® bioluminescence assay. *Environ. Toxicol. Chem.* **15**, 1730–1737.
60. Mendes, L. F., L. Zambotti-Villela, P. Colepicolo, E. Marinho-Soriano, C. V. Stevani and N. S. Yokoya (2013) Metal cation toxicity in the alga *Gracilaria domingensis* as evaluated by the daily growth rates in synthetic seawater. *J. Appl. Phycol.* doi:10.1007/s10811-013-0036-1.
61. Bermudes, D., V. L. Gerlach and K. H. Nealson (1990) Effects of culture conditions on mycelial growth and luminescence in *Panellus stypticus*. *Mycologia* **82**, 295–305.
62. Weitz, H. J., A. L. Ballard, C. D. Campbell and K. Killham (2001) The effect of culture conditions on the mycelial growth and luminescence of naturally bioluminescent fungi. *FEMS Microbiol. Lett.* **202**, 165–170.
63. Bermudes, D., M. E. Boraas and K. H. Nealson (1991) In vitro antagonism of bioluminescent fungi by *Trichoderma harzianum*. *Mycopathologica* **115**, 19–29.
64. Gadd, G. M. (1983) The use of solid medium to study effects of cadmium, copper and zinc on yeasts and yeast-like fungi: Applicability and limitations. *J. Appl. Microbiol.* **30**, 2155–2197.
65. Newman, M. C. and W. H. Clements (2008) *Ecotoxicology: A Comprehensive Treatment*. CRC Press, Boca Raton, FL.
66. Mendes, L. F., E. L. Bastos and C. V. Stevani (2010) Prediction of metal cation toxicity to the bioluminescent fungus *Gerronema viridilucens*. *Environ. Toxicol. Chem.* **29**, 2177–2181.
67. Steinberg, S. M., E. J. Poziomek, W. H. Engelmann and K. R. Rogers (1995) A review of environmental applications of bioluminescence measurements. *Chemosphere* **30**, 2155–2197.