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Jara Hurtado-Gallego[1], Keila Martín-Betancora[1], Ismael Rodea-Paloamares[1], Francisco Leganés[1], Roberto Rosal[2], Francisca Fernández-Piñas[1*]

[1] Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain

[2] Departamento de Ingeniería Química, Universidad de Alcalá, E-28871 Alcalá de Henares, Madrid, Spain

* Corresponding authors: francisca.pina@uam.es

Abstract

This work describes the construction of two novel self-luminescent bioreporter strains of the cyanobacterium *Nostoc* sp. PCC 7120 by fusing the promoter region of the *sodA* and *sodB* genes (encoding the superoxide dismutases MnSod and FeSod, respectively) to *luxCDABE* from *Photorhabdus luminescens* aimed at detecting pollutants that generate reactive oxygen species (ROS), particularly O_2^-. Bioreporters were tested against methyl viologen (MV) as the inducer of superoxide anion (O_2^-). Both bioreporters were specific for O_2^- and Limits of detection (LODs) and Maximum Permissive Concentrations (MPCs) were calculated: *Nostoc* sp. PCC 7120 pBG2154 (*sodA*) had a range of detection from 400 to 1000 pM of MV and for *Nostoc* sp. PCC 7120 pBG2165 (*sodB*) the range of detection was from 500 to 1800 pM of MV after 5 h-exposure. To further validate the bioreporters, they were tested with the emerging pollutant Triclosan which induced bioluminescence in both strains. Furthermore, the bioreporters performance was tested in two real environmental samples with different water matrix complexity, spiked with MV. Both bioreporters were induced by O_2^- in these environmental samples. In the case of the river water sample, the amount of bioavailable MV as calculated from the bioreporters output was similar to that nominally added. For the waste water sample, the bioavailable MV concentration detected by the bioreporters was one order of magnitude lower than nominal. These differences could be due to MV complexation with organic matter and/or co-occurring organic contaminants. These results confirm their high sensitivity to O_2^- and their suitability to detect oxidative stress-generating pollutants in fresh-waters.

Keywords: Bioluminescence; Cyanobacterial bioreporter; Superoxide dismutases; Superoxide anion; Mn-sod; Fe-sod

1. Introduction

The industrial development, origin of the modern societies, has brought as a consequence the deterioration of the natural systems. In aquatic ecological systems, one of the major sources of alterations is the continuous emission of pollutants of different kinds derived from human vital activity in cities and industrial activities. Thus, aquatic environments continuously receive through the treatment plants large amounts of pollutants whose effects are in most cases only partially known (Livingstone, 1998). Recent evidence on mechanistic studies indicate that oxidative stress is involved in most environmental toxicity processes (Livingstone, 2001; Valavanidis et al., 2005; Stone and Donaldson, 2006; Lushchak, 2011).

"Oxidative stress is an unavoidable by-product of the aerobic lifestyle" (Storz and Imlay, 1999). Aerobic organisms have developed defenses against the damaging effects of reactive oxygen species (ROS), intermediates caused by the reduction of oxygen. Oxidative stress is caused by a unbalance between ROS and the defenses against them in an organism (Latifi et al., 2009). These ROS can produce several damages in proteins, lipids and even in DNA. Different types of ROS exist in the organism, but the most important are O_2^-, hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and peroxynitrites. The hydroxyl radical is the most toxic; it has limited diffusion but can react with other molecules and inactivate them. O_2^- and hydrogen peroxide are less toxic but are implicated in the Fenton reaction ($Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + \bullet OH + OH^-$) that by producing the hydroxyl radical may cause highly damaging effects. Both ROS (O_2^- and H_2O_2) do not directly cause damages in DNA and they are not considered mutagens. However, by Fenton reaction, $\bullet OH$ is produced, indirectly causing genotoxic damages (Imlay, 2003). Normally in the cell, ROS levels are not toxic because there exist different ways to control them. One is energy dissipation by the high light-inducible proteins (HLIPs) which have different functions, for example quenching of singlet oxygen and non-photochemical quenching of absorbed energy (Komenda and Sobotka, 2016); the second one are nonenzimatic antioxidants like α-tocopherol or carotenoids; and the last one to control the level of ROS are the antioxidant enzymes (Latifi et al., 2009). For example, the O_2^- generated inside the cell is quickly

removed by superoxide dismutase (Sod) enzyme. In a similar way, other enzymes also remove H_2O_2, such as catalases or peroxiredoxins (Latifi et al., 2009). In the case of *Escherichia coli* (*E. coli*) the presence of peroxides like H_2O_2, provokes a peroxide stress response and the oxidative-stress-sensitive locus *oxyR* is induced, while in the case of superoxide stress response, the *soxRS* genes, are induced in the presence of $O_2^{\cdot-}$ (Robbens et al., 2010).

In the case of photosynthetic organisms such as cyanobacteria, the ROS present come from both, aerobic respiration and photosynthetic activity. Cyanobacteria are gram-negative bacteria, and they are known to survive a wide spectrum of environmental stresses. They are the only prokaryotic organisms that carry out an oxygen-evolving photosynthesis. As primary producers with a key role in the N and C cycles, they are a dominant component of marine and freshwater phytoplankton and are well suited for detecting contaminants in aqueous samples (Bachmann, 2003; Rodea-Palomares et al., 2009). Cyanobacteria, during their evolution, have survived every natural and human stresses and for this reason they are good organisms to study the response to a variety of environmental stresses (Banerjee et al., 2013). These organisms are constantly producing oxygen under illumination, so it is very important for them to prevent the electron escape from normal electron transfer pathways to oxygen, avoiding oxidative stress as much as possible (Latifi et al., 2009). Four types of Sods have been found in cyanobacteria: Cu-ZnSod, MnSod, FeSod and NiSod (Zhao et al., 2007; Priya et al., 2015). In case of filamentous heterocystous cyanobacteria like *Nostoc* sp. PCC 7120, both MnSod (encoded by *sodA*) and FeSod (encoded by *sodB*) co-exist (Raghavan et al., 2011). The cellular localization of these proteins has been studied in *Nostoc* sp. PCC 7120, where the MnSod is membrane bound and the FeSod is cytosolic (Li et al., 2002; Regelsberger et al., 2004). This different localization could be responsible for different roles in protecting the cells against oxidative damage. Specifically, the thylakoid membrane bound MnSod could imply a direct antioxidant activity, related with the photosynthesis.

Whole-cell bioreporters are cells genetically engineered to produce a dose-dependent measurable signal in response to chemical or physical agents in their environment (Harms et al., 2006; van der Meer and Belkin, 2010). Due to their ecological relevance, low cost and easy maintenance, cyanobacteria have been used for whole-cell bioreporter development which has demonstrated to be useful to assess toxicity in photosynthetic organisms (Shao et al., 2002; Rodea-Palomares et al., 2009, 2010, 2016), nutrient bioavailability in fresh waters (Schreiter et al., 2001; Bullerjahn et al., 2010; Munoz-Martin et al., 2011, 2014a, 2014b) as well as specific families of pollutants like heavy metals (Erbe et al., 1996; Peca et al., 2008; Martin-Betancor et al., 2015). At present, there exist whole-cell bioreporters for oxidative stress detection mostly based on recombinant heterotrophic bacteria such as *E. coli* (Belkin et al., 1996; Gu et al., 2004; Nizai et al., 2007, 2008; Ivask et al., 2010; Woutersen et al., 2011; Ooi et al., 2015). Despite the complex and interesting genetic regulation of oxidative stress in cyanobacteria (Latifi et al., 2009), at present no cyanobacterial whole cell bioreporters for oxidative stress exist.

The goal of this study was the construction of two cyanobacterial bioreporters, which detect O_2^- based on *Nostoc* sp. PCC 7120 (formerly known as *Anabaena* sp. PCC 7120). The constructed strains were named as *Nostoc* sp. PCC 7120 pBG2154 (based on *sodA*) and *Nostoc* sp. PCC 7120 pBG2165 (based on *sodB*). Once the strains were constructed, they were characterized using methyl viologen (MV). MV is an herbicide widely used in Europe until 2007, when the European Union banned its use because of its high toxicity. MV acts directly on photosynthesis at the level of photosystem I by intercepting electrons ranging from ferredoxin to $NADP^+$(Lascano et al., 2012). Because its action is specific to the photosynthetic machinery, and it is directly linked to the production of O_2^-, it is considered a model pollutant of oxidative stress in photosynthetic organisms (Lascano et al., 2012). The characterization of sensitivity profile was performed with increasing concentrations of MV in cyanobacterial culture medium AA/8 + N as well as in real waters spiked with MV. Furthermore, both strains were tested both with H_2O_2 to ensure their specificity for O_2^- and with Triclosan, an emerging pollutant model that produce ROS, specifically O_2^-, in photosynthetic organisms (González-Pleiter et al., 2017).

2. Materials and methods

2.1. Biological materials and culture conditions

Nostoc sp. PCC 7120 wild type was grown in AA/8+N culture medium (Table S1) at 28 °C with continuous illumination, at 60 μmol photons m^2 s^{-1} on a rotatory shaker in 250 mL Erlenmeyer flasks. Culture medium was supplemented with spectinomycin (Sp) (2 μg/mL) for the transformed strains *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165.

2.2. Construction of the self-luminescent O_2^- whole cell cyanobacterial bioreporters

The cyanobacterial whole-cell bioreporters for O_2^- detection were constructed using *Nostoc* sp. PCC 7120 wild type as host cell. The *luxCDABE* promoterless operon from *Photorhabdus luminescens* was obtained by digesting pBG2106 (Muñoz-Martín et al., 2011) with *kpn*I/*Sac*I and ligating to *Kpn*I/*Sac*I sites of pUC19 producing the plasmid pBG2129. pDU1 replicon from the filamentous, nitrogen-fixing cyanobacterium *Nostoc* sp. PCC 7524 (Walton et al., 1992) was obtained by digesting pRL1049 (Fernández-Piñas and Wolk, 1994) with *Bam*HI and ligating to *Bam*HI site of pRL1342 [Cmr, Emr, a RSF1010 based plasmid (Lázaro et al.,

2001)] producing plasmid pBG2140. pBG2129 was digested with *Kpn*I/*Sac*I and ligated to *Kpn*I/*Sac*I sites of pBG2140 to construct the plasmid pBG2142. To ensure a minimal baseline luminescence, the T4 transcriptional repressor was obtained by PCR amplification plus a restriction site for *Pst*I from the pHP45 plasmid (Prentki and Krisch, 1984). The product of PCR amplification was digested with *Pst*I and ligated to *Pst*I site of pBG2142 producing pBG2146.

To amplify the promoters from *sodA* and *sodB* genes, the primers described in Fig. S1 were used. These primers amplify these promoters plus a restriction sites for *Kpn*I/*Sal*I. The products of the PCR amplification were digested with *Sal*I and *Kpn*I and cloned in these sites in pBG2146, generating the plasmids: pBG2154 (Fig. S1A), harboring the *sodA*promoter and pBG2165 (Fig. S1A), harboring the *sodB*promoter. The resulting plasmids pBG2154 and pBG2165 contain a *PsodA*::*luxCDABE* and *PsodB*::*luxCDABE* transcriptional fusion where the *luxCDABE* operon is regulated under the control of the O_2^- -inducible promoter region of *sodA* and *sodB*, respectively. The integrity of the constructions in *E. coli* were confirmed by restriction analysis and DNA sequencing. The plasmids pBG2154 and pBG2165 were introduced into *Nostoc* sp. PCC 7120 wild type by conjugation as previously described (Elhai and Wolk, 1988; Elhai et al., 1997). The integrity of the transformation in *Nostoc* sp. PCC 7120 wild type was confirmed by PCR.

2.3. Chemicals

For the molecular cloning, enzymes required were from Takara and Fermentas. The kits used for plasmid extraction and purification were from Qiagen and Promega. The O_2^- inducing agent used in this study was methyl viologen (MV) (Aldrich, 98%). Besides, to test the specificity of the bioreporters to O_2^-, hydrogen peroxide (H_2O_2) (Panreac 30%) was also used. Stock solutions of MV and H_2O_2, were prepared with distilled water at 2.5 mM and 50 nM, respectively and were stored at 4 °C in darkness until used. The Triclosan (Sigma) stock was prepared with ethanol 70% at 100 ppm and stored at room temperature and darkness.

2.4. Bioluminescence assays

The response of recombinant bioluminescent cyanobacterial bioreporters *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 were characterized with MV (O_2^-producer) in the concentration ranges of 100–2000 pM; H_2O_2 (for testing the specificity) in the concentration ranges of 100–1000 nM and with Triclosan (for a further validation) in the concentration ranges of 1.7–5.1 nM.

Before the exposure experiments all the cyanobacterial bioreporters were grown until reaching the mid-log phase (OD_{750nm} = 0.5–0.6) because this growth stage was found to be the optimal for bioluminescent assays (data not shown) and were washed twice in AA/8 + N medium. For standardization purposes, cells were resuspended in fresh specific medium (AA/8 + N) at a final OD_{750nm} = 0.5 (Rodea-Palomares et al., 2009). Exposure experiments were performed in transparent 24 well microplates in a 1.5 mL final volume (Martin-Betancor et al., 2015). The different chemicals (MV, H_2O_2 and Triclosan) were added to the wells to get the desired final concentrations.

Plates were incubated at 28 °C in light (60 μmol m^2s^{-1}) on a rotary shaker up to 18 h. For the luminescence measurements, 100 μl of cell suspensions were transferred to an opaque 96-well microplate and luminescence recorded every 1 min for 10 min in a Centro LB960 luminometer (Berthold Technologies GmbH and Co. KG, Bald Wilbad, Germany) and the maximum record (usually at 8 min) was taken. Luminescence recordings were made after 1, 3, 5, 7, 10 and 18 h in the case of the MV experiments and after 15 min, 1,2,3, 4, 6, 8 and 24 h in the case of Triclosan.

Data are expressed as Bioluminescence Induction Factor (BIF) calculated by dividing the mean luminescence signal of a treated sample by the mean luminescence signal of the untreated sample. The limits of detection (LODs) which are the lowest MV concentrations with significant differences with respect to the untreated control and the maximum permissible concentrations (MPCs), which are the highest MV concentrations that do not cause toxicity to the organism, were determined (see Statistical Analysis). The luminescence was measured without supplementation of exogenous aldehyde as the strains harbored the *luxCDABE* and endogenously generated aldehyde was not limiting (as tested by the addition of exogenous aldehyde, data not shown).

2.5. Spiking experiments: performance of the bioreporters in environmental matrices artificially contaminated with MV

Two environmental water samples with different matrix composition were selected to validate the response of the O_2^-bioreporters under real environmental conditions. One of the water samples was from Guadalix River (Glx1). This is a tributary of the larger Jarama River and is located in central Spain, near the city of Madrid. Glx1 sampling point is located near the head waters at 1500 m and does not have anthropogenic influence. In addition we also selected a wastewater sample collected from an effluent of the secondary clarifier of the Alcalá de Henares wastewater treatment plant (WWTP) (Rosal et al., 2010b; Barrán-Berdón et al., 2011). This is the main WWTP in the region and it discharges treated wastewater effluents into the Henares River. The water samples manipulation, storage and analysis were performed essentially as previously described (Rodea-Palomares et al., 2010; Munoz-Martin et al., 2011; Martín-Betancor et al., 2015). The main physicochemical characteristics are described in Table S2.

For the MV spiking experiments, the water samples were supplemented with AA/8 + N, to ensure that any change in luminescence was not due to any nutrient deficiency. Cells were added to the environmental water samples already spiked with MV, to reach a final OD_{750nm} = 0.5 and 150 µL ten-fold concentrated medium was added in a final volume of 1.5 mL. Plates were incubated at 28 °C in light (60 µmol m^2 s^{-1}) on a rotary shaker. The measurements were performed after 5 and 7 h of exposure. The water samples were supplemented with AA/8+N growth medium, to ensure that any change in luminescence was not due to any nutrient deficiency. MV concentrations tested were in the range 200–10000 pM. For the luminescence measurements, 100 µL of cell suspensions were transferred to an opaque 96-well microplate and measured as described previously. Triplicate samples within each experiment were measured in at least three independent experiments.

2.6. Bioanalytical procedures

All data were obtained from a minimum of three independent experiments with three replicates for each assay situation. All test of statistically significant differences between data sets were performed using Student's *t*-test and one-way analysis of variance (ANOVA) which were computed using R analysis package (R for windows, 3.0.2 copyright© The Foundation for Statistical Computing).

3. Results

3.1. Characterization of the response of *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 to MV and H_2O_2

MV was selected as model pollutant to detect O_2^- since its mechanism of action, as continuous source of O_2^-, is well known. This herbicide affects directly photosystem I intercepting the electrons which go from ferredoxin to $NADP^+$ (Lascano et al., 2012). Therefore, the response of *Nostoc* sp. PCC 7120 pBG2154 (*sodA*::*luxCDABE*) and *Nostoc* sp. PCC 7120 pBG2165 (*sodB*::*luxCDABE*) to MV exposure was studied as a function of dose and exposure time. Fig. 1 shows the induction profiles of *Nostoc* sp. PCC 7120 pBG2154 (Fig. 1A) and *Nostoc* sp. PCC 7120 pBG2165 (Fig. 1B) for a range of MV concentrations (100 pM–2000 pM) at exposure times up to 18 h (BIF values, are calculated from the raw bioluminescence data shown in Fig. S2). As shown in Fig. 1A and B, BIF values increased in a dose-dependent manner as a function of MV concentration and exposure time. After 3 h of exposure a significant induction (ANOVA, Tukey's HSD $p < 0.05$) was achieved with all the concentrations above 400 pM in *Nostoc* sp. PCC 7120 pBG2154 and in *Nostoc* sp. PCC 7120 pBG2165 a significant induction (ANOVA, TukeyHSD $p < 0.05$) was observed with 500 pM of MV. However for both bioreporters a significant induction (ANOVA, Tukey's HSD $p < 0.05$) were achieved after 5 h of exposure. *Nostoc* sp. PCC 7120 pBG2154 LOD was 400 pM of MV, while for *Nostoc* sp. PCC 7120 pBG2165 the LOD was 500 pM of MV. In the former case, the detection range added up to 1000 pM MV (MPC), higher MC concentrations caused toxicity as they decreased bioluminescence. In the case of *Nostoc* sp. PCC 7120 pBG2165, the MPC was reached at 1800 pM. LODs and MPCs are summarized in Table 1. Table 1 also shows the main parameters of the regression curves in the linear ranges of the bioreporters response.

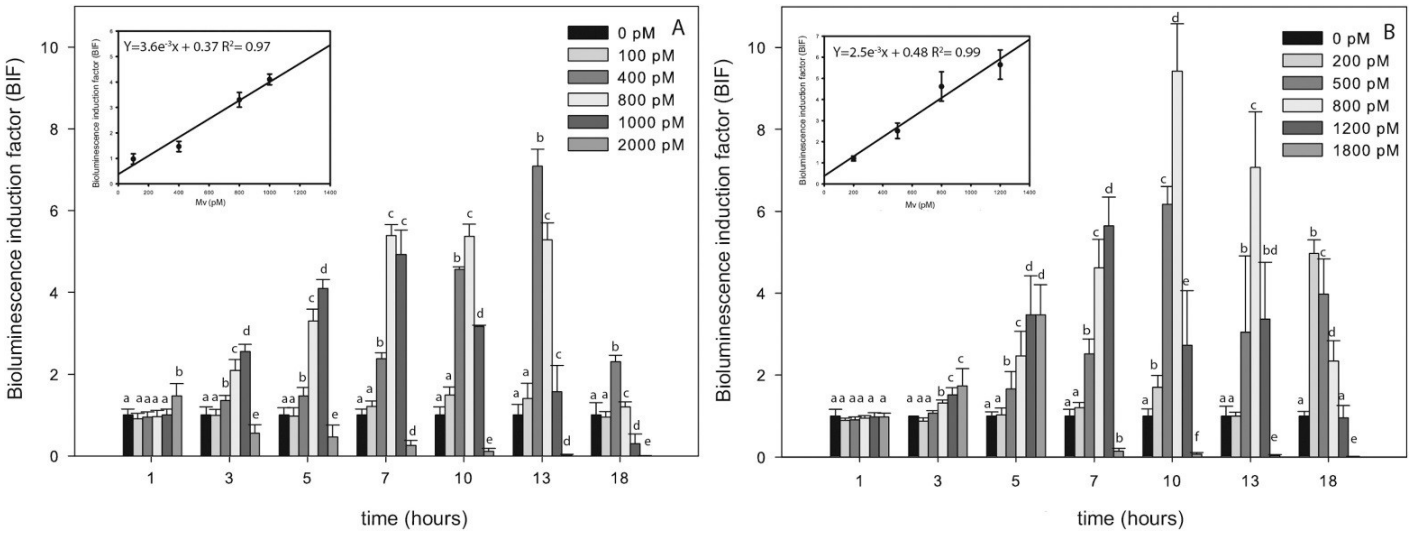

Figure 1. Response of *Nostoc* sp. PCC7120 pBG2154 (A) and of *Nostoc* sp. PCC7120 pBG2165 (B) to MV concentrations. Error bars indicate the standard error of the means from at least three independent experiments with three replicate samples. Each figure (A) and (B) contains an insert with the regression curve after 5 h and 7 h of exposure, respectively. P. Values with the same superscript letter were not significantly different (ANOVA, P < 0.05) within each time assayed.

Table 1. Limits of detection (LODs), maximum permissive concentrations (MPCs), regression equations and corresponding R^2 values for *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 for MV.

	LOD (pM)	MCPs (pM)	Equation
Nostoc sp. PCC 7120 pBG2154	400	1000	$Y = 3.6e^{-3}x+0.37$ ($R^2 = 0.9765$)
Nostoc sp. PCC 7120 pBG2165	500	1800	$Y = 2.5e^{-3}x+0.48$ ($R^2 = 0.9900$)

Although both bioreporters showed similar detection ranges, there were several differences between them. The maximum BIF for *Nostoc* sp. PCC 7120 pBG2154 was near 8-fold induction after 13 h of exposure with 400 pM of MV (Fig. 1A) while the maximum BIF for *Nostoc* sp. PCC 7120 pBG2165 was the highest of both strains, near 10-fold induction after 10 h of exposure with 800 pM of MV (Fig. 1A). Furthermore, after 10 h of exposure, *Nostoc* sp. PCC 7120 pBG2165 was significant induced by 200 pM of MV increasing the LOD of this strain.

In order to determine the specificity of these strains to O_2^-, the response of both bioreporters was also evaluated towards H_2O_2 exposure. The concentrations of H_2O_2 used ranging from 200 pM to 1 mM and the response of the bioreporters was measured along 24 h of exposure. No induction of bioluminescence was statistically significant (ANOVA, Tukey's HSD $P <$ 0.05) by exposure to H_2O_2 in any of the strains (Fig. S3 A and S3 B), determining the specificity of these bioreporter strains to O_2^-.

To further validate the bioreporters, they were tested with an emerging pollutant, Triclosan which is known to induce the formation of ROS, specifically O_2^- (Binelli et al., 2009; González-Pleiter et al., 2017; Park et al., 2017). Fig.2 A and 2 B shows *Nostoc* sp. PCC7120 pBG2154 and *Nostoc* sp. PCC7120 pBG2165 bioluminescence induction respectively, after 15 min, 1, 2, 3, 4, 6, 8 and 24 h of exposure to Triclosan. At shorter times (15 min and 1 h), bioluminescence induction was achieved in both bioreporters only with the highest tested concentrations (starting at 3.4 nM) of Triclosan. Maximum BIF of *Nostoc* sp. PCC7120 pBG2154 after 15 min and 1 h of exposure with 5.1 nM Triclosan was near three-fold and the maximum BIF of *Nostoc* sp. PCC7120 pBG2165 was near four-fold (Fig. 2 A and 2 B respectively). After 2 h, the observed induction with the highest concentrations tested started to decline and was almost negligible after 24 h of exposure probably due to increased toxicity. However, after 24 h of exposure a significant induction was observed for both bioreporters for the lower concentrations used (1.72 and 2.58 nM); maximum BIF of *Nostoc* sp. PCC7120 pBG2154 with 1.72 nM of Triclosan was near ten-fold and the maximum BIF of *Nostoc* sp. PCC7120 pBG2165 with 2.58 nM of Triclosan was near five-fold. This was probably due to delayed cellular detection and sensing of these lower concentrations of Triclosan.

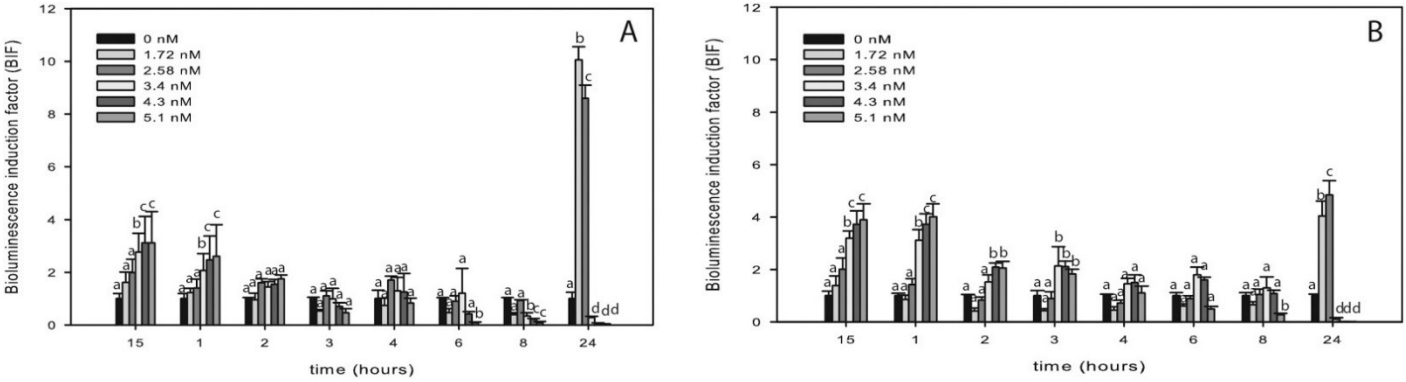

Figure 2. Response of *Nostoc* sp. PCC7120 pBG2154 (A) and of *Nostoc* sp. PCC7120 pBG2165 (B) to increasing Triclosan concentrations after 15 min, 1, 2, 3, 4, 6, 8 and 24 h of exposure. Data represent the mean ± standard deviation of at least three independent experiments. P. Values with the same superscript letter were not significantly different (ANOVA, $P < 0.05$) within each time assayed.

3.3. Response of *Nostoc* sp. PCC7120 pBG2154 and *Nostoc* sp. PCC7120 pBG2165 bioreporters to MV in environmental samples: spiking experiments

To test the suitability of *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 as bioreporters of O_2^- in environmental samples, we exposed them to MV in two different water matrices. One of these samples was from upstream of Guadalix River (Glx1), while the other one was from an effluent of a secondary clarifier of a WWTP. Glx 1 represents a near pristine water with low electrical conductivity, PO_4^{3-}-P, alkalinity, NO_3-N and NH_4^+-N, while WWTP presents increasing anthropic influence represented by increasing values for those parameters (Table S2). Both waters (Glx1 and WWTP) were previously studied and no herbicide was found using LC-MS according to the procedure described elsewhere (Rosal et al., 2010b; González-Pleiter et al., 2013). However, WWTP water sample presented traces of emerging pollutants such as pharmaceutical pollutants (Rosal et al., 2010b; González-Pleiter et al., 2013). For the experiments, water matrices were artificially contaminated with

increasing MV concentrations, and bioavailable MV concentrations were estimated from the calibration curves presented in Table 1. Table 2 shows the estimates of the bioavailable MV concentrations as measured by *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 after 5 h and in some cases, 7 h of exposure.

Table 2. Nominal MV concentrations tested in water samples in the spiking experiments and the bioreporters output after 5 h of exposure (calculated from Figs. 1A and B) in water samples for two sampling points (Glx1 in the upstream course of the Guadalix River and WWTP from the effluent of Alcalá de Henares WWTP).

Samples	MV nominal (pM)	MV bioavailable by *Nostoc* sp. PCC 7120 pBG2154 (pM)	MV bioavailable by *Nostoc* sp. PCC 7120 pBG2165 (pM)
Glx1	200	Nd	Nd
	600	514 ± 11	585.8 ± 9.2
	1000	669 ± 26	1198 ± 15
WWTP	200	Nd	Nd
	400	Nd	Nd
	600	Nd	Nd
	800	Nd	Nd
	1000	Nd	Nd
	1200	Nd	Nd
	2000	Nd	Nd
	5000	Nd	481[a] ± 49
	10000	1034[a] ± 20	1077[a] ± 61

Nd: not detected.

[a] 7 h exposure. Calibration curves after 7 h of MV exposure to *Nostoc* sp. PCC 7120 pBG2154: $Y = 4.75\ e^{-3}\ x + 0.74$ and to *Nostoc*sp PCC 7120 pBG2165: $Y = 4.6e^{-3}x + 038$.

In Glx1 matrix, nominal and bioavailable MV concentrations (estimated based on the calibration curve of the bioreporter) were in the same order of magnitude, indicating that the bioreporters were able to detect nearly all the added MV as bioavailable, but the bioreporter *Nostoc* sp. PCC 7120 pBG2165 detected slightly more MV bioavailable than *Nostoc* sp. PCC 7120 pBG2154. By the contrary, there was no bioluminescence induction at the same MV concentrations (200–1000 pM) when MV was added to the WWTP water matrix (Table 2). Near one order of magnitude higher concentrations of nominal MV (5–10 nM) and an exposure time of 7 h were required for bioluminescence induction of the bioreporters. In both cases, the MV concentrations detected by the bioreporters were one order of magnitude lower than the nominally added. In both water samples, the bioreporter *Nostoc* sp. PCC 7120 pBG2165 was able to detect slightly more MV bioavailable concentration than the bioreporter *Nostoc* sp. PCC 7120 pBG2154.

4. Discussion

This study presents the construction of two novel self-bioluminescent bioreporters of *Nostoc* sp. PCC 7120 named *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165, which respond to oxidative stress (specifically to O_2^-). The agent responsible for causing oxidative stress and hence the induction of luminescence is MV, an herbicide that generates O_2^-, previously used in studies of oxidative stress (Belkin et al., 1996; Nizai et al., 2008; Ivask et al., 2010).

As reported before, the LODs of the cyanobacterial bioreporters were very similar (400 pM and 500 pM after 5 h of exposure). Furthermore, after 10 h of exposure, the bioreporter *Nostoc* sp. PCC 7120 pBG2165 was able to detect 200 pM of MV, increasing the sensitivity of this bioreporter. These differences may be attributed to the different localization of both Sods in the organism; to date, *E. coli* has been the most used bacterium for the construction of oxidative stress bioreporters to study water pollution (Robbens et al., 2010). This organism possesses two regulators SoxRS and OxyR which induce the expression of antioxidant activities in response to O_2^- and H_2O_2 stress, respectively. The SoxRS-regulated genes include *SodA, nfo, zwf, micF*, *pqi*5 gene and OxyR-regulated *katG*, *gor*A and *dps* genes (Storz and Imlay, 1999; Robbens et al., 2010). Comparing these results of the cyanobacterial strains with previous studies of the oxidative stress bioreporter strains based on other bacteria (Table 3), it could be concluded that the bioreporter strains *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 are good bioreporters as they are very sensitive and the optimum time for their induction is not too long. For example, Belkin et al. (1997) constructed an *E. coli*-based bioreporter strain carrying the *micF*:: *luxCDABE* [*micF* encodes an outer membrane porin regulator (Robbens et al., 2010)] fusion, also using MV as bioluminescence inducing agent, reporting a bioreporer LOD of 0.01 mg/L (4×10^4 pM), (Table 3). Nizai et al. (2008) also constructed a *E. coli* oxidative stress bioreporter strain by fusing the *pgi*gene (encoding Glucose-6-phosphate isomerase) to the *luxCDABE* operon. They found that the LOD was 0.6 mg/L (24×10^5 pM) (Table 3). The promoter of the genes *zwf* and *fpr* of *E. coli* were fused to l*uxCDABE* operon and the maximum induction of bioluminescence with MV were 9.7 mM and 77.8 mM respectively (LODs were not reported) (Nizai et al., 2007) (Table 3). Lee and Gu (2003) constructed a *E. coli* based bioreporter by fusing *sodA* to *luxCDABE* whose LOD was 0.015 mg/L (6×10^4 pM) of MV (Table 3). Arias-Barreiro et al. (2010) used an *E. coli* strain with ro-GFP2 which emits fluorescence with increasing concentrations of menadione (model of O_2^- producer) with a LOD of 5.8×10^4pM, the strain was sensitive to oxidative stress produced by arsenite and selenite. In the case of the strain K12:*soxRSsodAlux,* based also in *E. coli* (Ivask et al., 2010) (Table 3), the authors fused *sodA* to *luxCDABE* and the LOD was 0.01 mg/L (3.8×10^4 pM) of MV, this LOD makes this bioreporter the most sensitive E. *coli*-based one to O_2^-. In the same study, they also tested the strain with menadione but the LOD was higher than that of MV

(Table 3). Apart from *E. coli* based bioreporters, Porteous et al. (2000) constructed a *Pseudomonas fluorescens* based on plasmid pUCD607 (where bioluminescence is directly correlated with metabolic activity) with a LOD of 7.7×10^7 pM of MV (Table 3). However, both cyanobacterial bioreporters were much more sensitive (between one and three orders of magnitude) to MV than any of those bacterial optic-based bioreporters (Table 3). The detection ranges of *Nostoc* bioreporters were narrow in comparison with the other bioreporters (Table 3) probably due to the high toxicity of O_2^- to cyanobacteria. This might be an issue, but the higher sensitivity of both strains may compensate this, and diluted samples could always be used. Concerning the assay time, it was similar for all tested bioreporters (Table 3).

The comparison between the results of the *Nostoc* sp. PCC 7120- based bioreporter and those of other bacterial strains, suggest that they could be complementary detecting pollutants which may cause oxidative stress; the lowest concentration of O_2^- could be detected by using the cyanobacterial bioreporters, while the highest levels could be measured by other bacterial-based bioreporters. These bioreporters could also be used to study mechanisms of action of different pollutants.

Table 3. Overview of bacterial optical bioreporter strains for the detection of O_2^-.

Strain	Organism	Specificity	Promoter/ Gene	LODs (pM)	Detection Range (pM)	Assay time (h)	References
DPD2515	*E coli.*	O_2^- H_2O_2	*micF*	4×10^4 (MV)	-	3	Belkin et al., 1997
PGRFM	*E coli.*	O_2^- H_2O_2	*pgi*	2.4×10^6 (MV)	2.4×10^6 - 2.4×10^9 (MV)	2	Nizai et al., 2008
EBHJ	*E coli.*	O_2^- H_2O_2	*sodA*	6×10^4 (MV)	6×10^4 - 6×10^7 (MV)	2	Lee and Gu, 2003
ZWF RFM_{443}	*E coli.*	O_2^-	*zwf*	9.7×10^9 (MV)	-	3-3.8	Nizai et al., 2008
FPR RFM_{443}	*E coli.*	O_2^-	*fpr*	7.78×10^{10} (MV)	-	3-3.8	Nizai et al., 2008
K12::*soxRSSodAlux*	*E coli.*	O_2^-	*sodA*	3.8×10^4 (MV) 5.78×10^6 (Menadione)	3.8×10^4 - 3.8×10^8 (MV) 5.78×10^6 - 5.78×10^7 (Menadione)	2-5 2.5	Ivask et al., 2007
pUCD607	*P.fluorescens*	O_2^-	*pUCD607*	7.7×10^7 (MV)	7.7×10^7 - 7.7×10^9 (MV)	24-48	Porteous et al., 2000
ro-GFP2	*E coli.*	O_2^-	*ro-GFP2*	5.8×10^4 (Menadione)	5.8×10^5 - 5.8×10^8 (Menadoine)	3.33	Arias-Barreiro et al., 2000
pBG2154	*N.* PCC 7120	O_2^-	*sodA*	400 (MV)	400-1000 (MV)	5	This study
pBG2165	*N.* PCC 7120	O_2^-	*sodB*	500 (MV)	500-1800 (MV)	5	This study

-: not reported.
[a] EC_{50}
[b] Maximum response
[c] LOEC.

One of the advantages of cyanobacteria with respect to the other bacterial strains is their autotrophic character, which gives them the ecological relevance of being primary producers in aquatic ecosystems. Therefore, any deleterious effect in these organisms, would affect the rest of the trophic chain. Consequently, these organisms have been used by our group to construct bioreporters of global toxicity, bioavailability of nutrients and heavy metal toxicity (Rodea-Palomares et al., 2009, 2010; Munoz-Martin et al., 2011, 2014a, 2014b; Martin-Betancor et al., 2015; Rodea-Palomares et al., 2016). In addition, as previously mentioned, cyanobacteria were the first to use oxygen photosynthesis on planet Earth, which helps to understand the great variety of adaptations (proteins that act against ROS) that they have to mitigate oxidative stress (Latifi et al., 2009). The genes chosen in this work, *sodA* and *sodB* encoding MnSod (located on the membrane of the thylakoids of *Nostoc* sp. PCC 7120) and FeSod (located in the cytoplasm), have already been studied by Raghavan et al. (2011) that verified that they responded to oxidative stress in the presence of MV. MnSod and FeSod have different role for the protection from oxidative stress in *Nostoc* sp. PCC 7120. MnSod may play a more important role in the protection of the cell under nitrogen-fixing conditions while FeSod may be more important under nitrogen-replete conditions (Banerjee et al., 2013). Despite these studies about MnSod and FeSod of

cyanobacteria, no bioreporters based on them have been reported before.

To test the specificity of the bioreporters, H_2O_2 (ROS but not via O_2^- production) was used. No induction was observed in any of the cyanobacterial bioreporters (Fig. S3) confirming the bioreporters specificity. Table 3 also shows the specificity (using H_2O_2 to test it) of the other bacterial-based bioreporters, although from the eight bioreporters, only six tested that. From these six, three responded to both O_2^- and H_2O_2 peroxide (Belkin et al., 1997; Lee and Gu, 2003; Nizai et al., 2008), while three of them responded only to O_2^- (Nizai et al., 2007; Ivask et al., 2010). Further validation of the bioreporters was to test them with the emerging pollutant Triclosan. Triclosan is known to induce the formation of ROS (Tamura et al., 2012; Han et al., 2016; González-Pleiter et al., 2017). This pollutant is thoroughly distributed in the environment and has been previously used (Rosal et al., 2010a; González-Pleiter et al., 2017) to study their persistence and toxicity in aquatic environments. In a study of González et al. (González-Pleiter et al., 2017), they used *Chlamydomonas reinhardtii* to investigate the early cellular response to Triclosan and among other toxics effects, they saw increased ROS formation, specifically O_2^-. Both bioreporters showed bioluminescence induction after exposure with Triclosan, so they can be used to detect O_2^- -inducing pollutants. It is interesting to note that a dual behavior was observed with increased induction at high concentrations of Triclosan at shorter exposure times, followed by a decline probably due to toxicity and later induction at longer times of exposure but only with the lower concentrations tested. This might be due to the time needed for the cells to detect and respond to increasing concentrations of this pollutant. The difference of the observed bioreporter induction to Triclosan and to MV may be due probably to the different chemical nature of both pollutants and mode of action. MV is an herbicide with a direct effect on photosystem I, while Triclosan is a wide spectrum antimicrobial agent that is able to produce oxidative stress in many organisms (Kawanai, 2011; Cherednichenko et al., 2012; González-Pleiter et al., 2017), so the pathways and timing to detect and respond to both pollutants might be different.

The bioreporters performance also has been tested with natural waters spiked with increasing concentrations of MV. The waters used reflect different complexities depending on the human influence. This approach has been previously used with bacterial bioreporters (Magrisso et al., 2008; Hynninen and Virta, 2009; Munoz-Martin et al., 2011, 2014a, 2014b). The bioavailable MV concentrations detected in the river water (Glx1) by both bioreporters, were in the same order of magnitude than the nominal concentration of MV added in the experiments. However in the presence of polluted water (WWTP), no induction of luminescence was shown for any strain at the same concentrations of the river water. Increasing the concentration of MV (5 and 10 nM), the bioreporters were induced, however, the bioavailable MV concentrations detected were one order of magnitude lower than those nominally added. One reason could be the formation of complexes between the MV and the organic matter and co-occurring contaminants. The formation of these complexes could decrease bioavailable MV and its capacity to produce O_2^-. This idea could be supported by the fact that MV is inactivated on active anionic surfaces and in contact with the clay particles of the soil (Fernández et al., 1998; World Health Organization, 2002).

5. Conclusions

Two novel bioreporters strains based on *Nostoc* sp. PCC 7120 have been constructed to detect oxidative stress (specifically O_2^-). *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 are the first bioreporters based on cyanobacteria that specifically detect oxidative stress whose limits of detection are 400 pM and 500 pM, respectively. Comparing these data with the oxidative stress bacterial-based bioreporter strains previously constructed, it is shown that the strains *Nostoc* sp. PCC 7120 pBG2154 and *Nostoc* sp. PCC 7120 pBG2165 are the most O_2^--sensitive bioreporter strains constructed to date. The two bioreporter strains are capable to detecting bioavailable MV in different real water samples and therefore, both bioreporters have been validated to detect oxidative stress-generating pollutants in fresh-waters.

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SUPPLEMENTARY MATERIAL

Two novel cyanobacterial bioluminescent whole-cell bioreporters based on superoxide dismutases MnSod and FeSod to detect superoxide anion

Jara Hurtado-Gallego[1], Keila Martín-Betancora[1], Ismael Rodea-Paloamares[1], Francisco Leganés[1], Roberto Rosal[2], Francisca Fernández-Piñas[1*]

[1] Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Madrid, Spain
[2] Departamento de Ingeniería Química, Universidad de Alcalá, E-28871 Alcalá de Henares, Madrid, Spain

* Corresponding authors: francisca.pina@uam.es

Contents

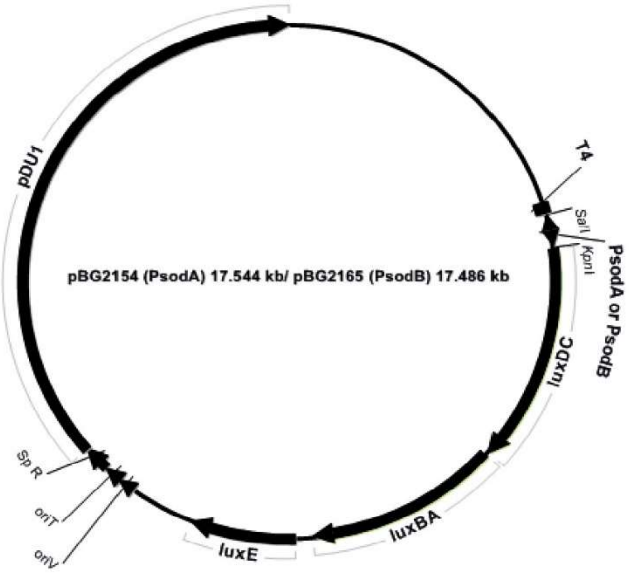
Table S1. Chemical composition of culture medium for *Nostoc* sp. PCC 7120 (AA/8+N).

AA/8 + N	
Element	**Concen. (µM)**
$MgSO_4$	125
$CaCl_2$	62.5
NaCl	500
K_2HPO_4	250
$NaNO_3$	2500
KNO_3	2500
Na_2EDTA	9.59375
$FeSO_4$	8.645
$MnCl_2$	1.13625
MoO_3	0.15625
$ZnSO_4$	0.095625
$CuSO_4$	0.0395
H_3BO_3	5.78125
NH_4VO_3	0.0245
$CoCl_2$	0.021

Table S2. Main physicochemical characteristics of environmental waters used in the study.

Physicochemical parameters	**Guadalix river (Glx1)**	**Alcalá Wastewater treatment plan (WWTP)**
Water temperature (ºC)	8.6	13
pH	6.9	7.5
Conductivity (µs cm^{-1})	100	702
PO^{3-4} –P (mgL^{-1})	0.05	1.1
Alkalinity (mg L^{-1} $CaCO_3$)	14.5	472
Hardness (mg L^{-1} $CaCO_3$)	17.7	176
N-NO_3^- (mg L^{-1})	0.2	7
N-NH_4^+ (mg L^{-1})	0.05	1.5

A



B

5´**TTCCGTCGACTGTATTTCCATTCAC**
……GTTTTGTATTTCCATTCAC
…..CAAAACATAAAGGTAAGTG …… ACTAAAGTACAACAACGGAGTT…
… TGATTTCATGTTGTTGCCTCTT…
TTTCGGTACCTGTTGTTGCCTCTT 3´

C

5´TGGAGTCGACAGCTTAAAAGTCTTATGC
……AGCTTAAAAGTCTTATGC
……TGAATTTTCAGAATACG …… ATGTCCATGGTAAAACACTTCAGGAGAG
TACAGGTACCATTTTGTGAAGTCCTCTC..
GTACAGGTACCATTTTGTGAAGTCCTCTC3´

Figure S1. Scheme of plasmids pBG2154 and pBG2165 construction. (A) pBG2154 and pBG2165 resulted of cloning a sequence containing the *sod*A and *sod*B promoter region, respectively, in kpnI/SalI site of pBG2146. Transcription of the *lux* operon in pBG2154 and pBG2165 is under the control of the *sod*A and *sod*B promoter (PsodA and PsodB), respectively. (B) and (C) Sequence of PCR primers (shown in bold) used to amplify the sodA and sodB promoter sequence, respectively including the kpnI/SalI restriction enzymes sequences (underlined).

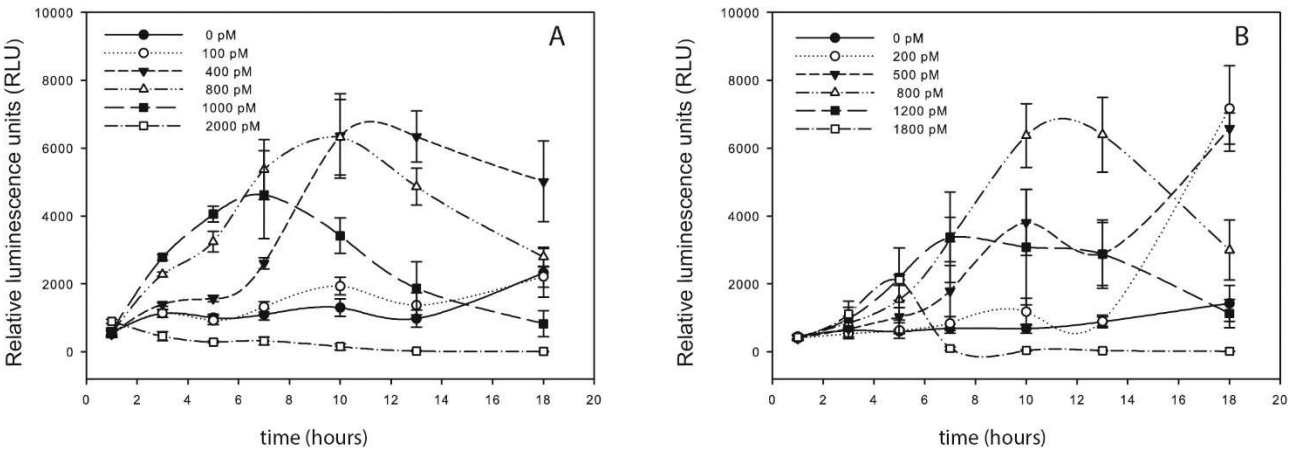


Figure S2.Raw induction data of *Nostoc* sp. PCC 7120 pBG2154 (A) and *Nostoc* sp. PCC7120 pBG2165 (B) exposed to increasing concentrations of MV, from which BIF were calculated.

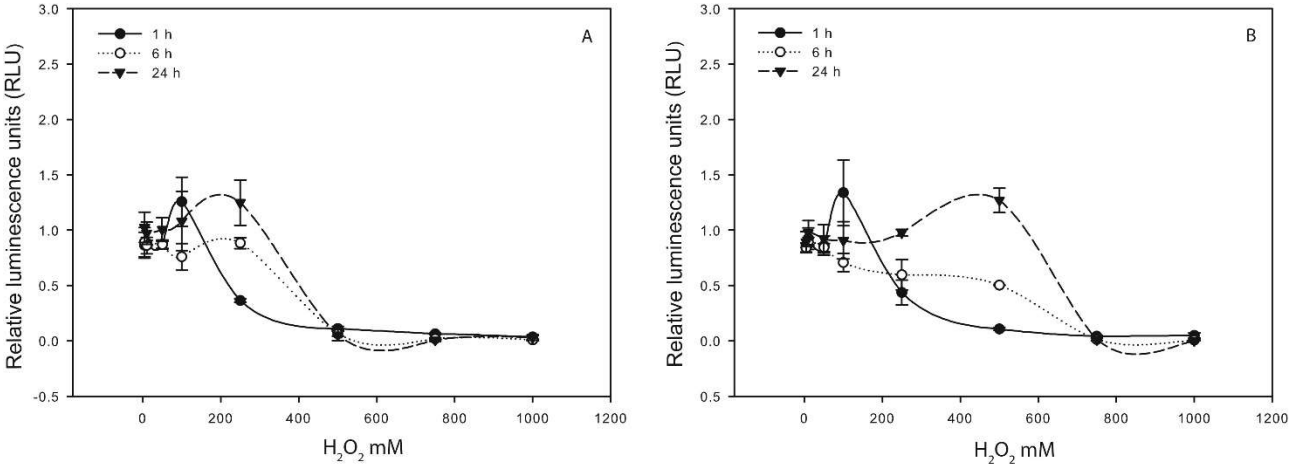


Figure S3. Response of *Nostoc sp*. PCC7120 pBG2154 (A) and of *Nostoc sp.* PCC7120 pBG2165 (B) to increasing H_2O_2 concentrations after 1 h (●), 6 h (○) and 24 h (▼) of exposure.