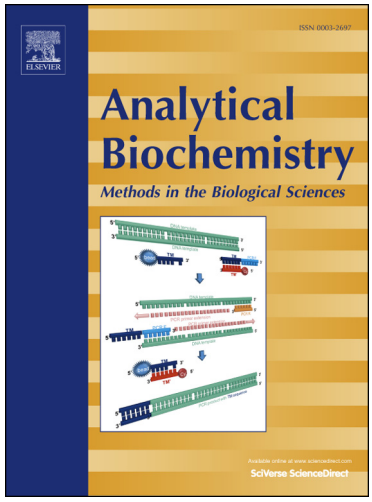

Suitability of *Macrolampis* firefly and *Pyrearinus* click beetle luciferases for bacterial *light off* toxicity biosensor

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Suitability of *Macrolampis* firefly and *Pyrearinus* click beetle luciferases for bacterial *light off* toxicity biosensor

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***Macrolampis* and *Pyrearinus* luciferases for biosensor**

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Table of Contents: enzymatic assays and analyses.

Abstract

Bioluminescence is widely used in biosensors. For water toxicity analysis, the naturally bioluminescent bacteria *Vibrio fischeri* have been extensively used. We investigated the suitability of two new beetle luciferases for *Escherichia coli light off* biosensors: *Macrolampis* firefly and *Pyrearinus termitilluminans* click beetle luciferases. The bioluminescence detection assay using this system is very sensitive being comparable or superior in to *Vibrio fischeri*. The luciferase of *Pyrearinus termitilluminans* produces a strong and sustained bioluminescence which is useful for less sensitive and inexpensive assays which require integration of the emission, whereas *Macrolampis* luciferase displays a flash-like luminescence which is useful for fast and more sensitive assays. The effect of heavy metals and sanitizing agents was analyzed. Zinc, copper, 1-propanol and iodide had inhibitory effects on bioluminescence and growth assays, however, in these cases the bioluminescence was not very reliable indicator of cell growth and metabolic activity, because these agents also inhibited the luciferase. On the other hand, mercury and silver strongly affected cell bioluminescence and growth but not the luciferase activity, indicating that bioluminescence was a reliable indicator of cell growth and metabolic activity in this case. Finally, bioluminescent *E. coli* immobilized in agarose matrix gave a more stable format for environmental assays.

Keywords: luciferase, biosensor, bioluminescence

Introduction

Biosensors are analytical devices that combine the recognition of a biological substance of interest and the transduction of the response in a measurable signal, allowing a quantitative and specific analysis [1, 2, 3, 4]. These devices are useful due to their easy preparation, minimum quantity of sample required, easy storage, good reproducibility, selectivity and sensitivity, relatively low cost and fast response time [1, 4]. Currently they are used also for environmental applications [5]. Furthermore, biosensors show the advantage of being useful to analyze bioavailability, in contrast to other physical-chemical analytical techniques.

Among different kinds of biosensors, bioluminescent biosensors are very fast, sensitive and gaining popularity. A bioluminescent biosensor relies on luciferases or photoproteins, which are enzymes that requires oxygen to oxidize luciferin, producing light in a quantitative fashion. Bioluminescent biosensors can be divided in enzymatic, in which the enzyme is immobilized and directly or indirectly used to detect a specific analyte, or whole cell biosensors, in which the luciferase is constitutively expressed or induced by a specific analyte. Whole cell biosensors can be further divided in *light off* and *light on* biosensors. In the *light off* biosensor luminescent is inhibited by specific agents affecting metabolism, reducing the intensity of the light signal. In the *light on* biosensors the luciferase gene is under control of an inducible promoter, being expressed in the presence of the inducer (specific analyte) [6, 7].

Whole cell bioluminescent biosensors can be immobilized [8], or suspended in a growth medium, and the bioluminescence is monitored by a device that measure the light intensity (luminometer) [9]. They are built by cell transformation with an expression vector containing the reporter gene under the control of an inducible promoter by specific or constitutive signals, expressing luciferase [10], and used to measure the toxicity of chemicals

in micromolar range (μM) in which bioluminescence intensity is reduced when cells make contact with a toxic or lethal factors [11].

Examples of the last category are heavy metal biosensors, where a reporter gene is under control of a heavy metal inducible promoter [6, 7, 12, 13, 14]. The luciferases from bacteria, fireflies and jelly fishes have been extensively used in a variety of biosensors, including redox metabolism, ATP analysis and calcium measurement. The bacterial and firefly luciferases have been used in whole cell biosensors and both luciferases rely on metabolic power to produce light, bacteria use $FMNH_2$ and firefly luciferase ATP. The naturally bioluminescent bacterium *Vibrio harvey* was the first whole cell *light off* biosensor used for general water toxicity analysis, using the Microtox™ kit [15, 16, 17, 18]. This kit has been used to analyze presence of toxic agents including mercury, zinc, copper, nickel and cadmium in water treatment plants [19, 20, 21].

Firefly luciferase has been one of the most extensively used enzymes for a wide range of bioanalytical purposes, including ATP assays, biomass estimation, enzyme assays, reporter gene assays, bioimaging, and biosensors [22]. However, other beetle luciferases with distinct bioluminescence properties such as spectrum, kinetics and pH-sensitivities are currently available but where not used for bioanalytical purposes. Beetle luciferases are functionally classified in pH-sensitive (Lampyridae), whose spectrum shifts from yellow-green to red in acid pH, high temperature and presence of heavy metals, and pH-insensitive (Elateridae and Phengodidae) which are not affected by these factors [23].

Here we analyzed the suitability of two new beetle luciferases cloned in our laboratory, a pH-sensitive and a pH-insensitive, for whole cell toxicity biosensing. The Brazilian larval click beetle *Pyrearinus termitilluminans* luciferase, which produces the most blue-shifted bioluminescence among beetle luciferases and is pH-insensitive, and

Macrolampis sp2 firefly luciferase, which produce a bioluminescence spectrum especially sensitive to pH, temperature and heavy metals.

Material and Methods

Reagents. D-luciferin, tris base (Promega, Madison, USA); adenosine tri phosphate (Ambresco, Ohio, USA); dithio-D-threitol (Thermo Fisher Scientific, Waltham, USA); ethylenediamine tetraacetic acid, magnesium sulfate (Synth, Diadema, Brazil); isopropyl β-D-1-thiogalactopyranoside (Fermentas, Burlington, ON, Canada); Triton-X-100 (Riedel-De Haen, Berlin, Germany); Luria Bertani broth and agar Miller (Himedia, Curitiba, Brazil); glycerin (Ecibra, São Paulo, Brazil); monosodium and disodium phosphate (Cromoline, Diadema, Brazil). Ultrapure water was used in all assays.

***E. coli* and plasmids.** *E. coli* ATCC was obtained from Oxoid Limited (England, UK). For expression of high levels of luciferases, *E. coli* BL21 (DE3) cells were used. The plasmids pMac and pPy, harboring the luciferase cDNA from *Macrolampis* sp2 and *Pyrearinus termitilluminans*, respectively, were previously constructed in our laboratory [24, 25].

Luciferase expression. Bacteria transformed with pMac and pPy were grown in LB medium/Ampicillin liquid cultures at 37° C until $O.D._{600}$ = 0.40, and then induced with 1 mM IPTG until $O.D._{600}$ = 1.5 at 20° C.

Luciferase extraction. Induced cells for luciferase expression were harvested by centrifugation at 2.500 g for 15 min at 4° C and resuspended in cold extraction buffer (25 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol), 1 mM 1,4-dithio-D-threitol (DTT) and protease inhibitor cocktail, at pH 8.0, freeze-thawed and sonicated (Misonix, Nova York, USA) five times and finally centrifuged at 15.000 g for 15 min at 4° C.

CCD imaging. Bioluminescence imaging of bacteria in liquid cultures (LB broth) or in semi-solid medium (Top Agar: LB broth with 0.7% (w/v) agarose) was done after adding 5 µL of 10 mM D-luciferin at pH 5.0 to 90 µL of cell suspension, exposing at different times and sensitivities, depending on the bioluminescence intensity of the sample, using a Light Capture CCD II Camera (ATTO, Tokyo, Japan).

Luminometric assays of *in vivo* and *in vitro* bioluminescence. Bioluminescence activities of liquid cultures of *E. coli* expressing beetle luciferases, and luciferase *in vitro* assays were measured using an AB2200 luminometer (ATTO, Tokyo, Japan). For *in vivo* bioluminescence of liquid cultures and suspensions, 10 µL of 10 mM D-luciferin pH 5.0 were mixed to 90 µL of culture in the luminometer tube, and intensity measured in *cps* (counts per second) during 10s. The effect of heavy metals and sanitizers on bioluminescence intensities was tested by mixing 500 µL of liquid cultures with 50 µL of heavy metals at 5,0 µg/mL or sanitizers, incubation during 1 h, and mixing 90 µL of the treated suspension with 10 µL of D-luciferin. Each experiment was repeated 3 times independently, each independent experiment of *in vivo* and *in vitro* bioluminescence was assayed in triplicate.

Bioluminescence spectra. Bioluminescence spectra were recorded in a Hitachi F4500 spectrofluorometer (Hitachi High-Technologies, Tokyo, Japan) with the excitation lamp off and the emission shutter open. The spectra were scanned from 450 nm to 700 nm at 2400 nm/min. For the *in vitro* bioluminescence, 50 µL of bacterial extracts were mixed with 400 µL of assay solution (10 mM luciferin, 40 mM ATP, 80 mM $MgS0_4$, in 0.10 M Tris-HCI, pH 8.0). Each spectra was the result of three independent assays.

Effect of divalent heavy metals on bacterial growth. The growth inhibition of bacteria was analyzed by the agar diffusion test [26] in which 2 mL of culture at 10^8 CFU/mL are mixed with warm Agar and added over solid Agar dishes. Then, 13 mm diameter absorbing paper dishes previously saturated with the sample to be analyzed were put over the bacteria/Agar

plates and incubated at 37° C during 15 h for bacterial growth and inhibition. The inhibition of growth is verified by the presence of a zone of inhibition (growth inhibition of microorganisms around the paper dishes due to diffusion of the sample).

Microbiological assays. To perform the microbiological assays, 50 µl of the suspension of *E. coli* transformed with pMac and pPy was added in a tube containing 9 ml of 0.9% (w/v) saline followed by decimal serial dilutions up to 10^{-5}. Then, 100 µl of each dilution was spread, in duplicate, on the surface of LB plates with ampicillin (1 mg/mL). The plates were incubated in an inverted position for 24 hours at 37° C for subsequent counting of title (CFU/mL) [27]. In order to evaluate the correlation between CFU/mL and absorbance the suspension underwent a new decimal serial dilution – 10^{8} to 10 – and its absorbance (O.D. 600 nm) was measured as previously described.

Tetrazolium colorimetric assay of bacterial viability. In an Elisa plate, 90 µL of *E. coli* at 10^{8} CFU/mL are mixed with 10 µL of sample to be analyzed and 10 µL of 2,3,5-triphenyltetrazolium chloride (TTC) at 1 mg/mL, and incubated at 37° C during 1 h. The tetrazolium reagent has no color, but when reduced, it gives a bright red color, indicating the presence of live bacteria [28].

Biolux assay. The toxicity assay using naturally bioluminescent bacteria *Vibrio fischeri* from the Biolux kit (Umwelt, Blumenau, Brazil) was performed after the reactivation of the lyophilized cells according to the manufacturer's instructions. After reactivation, the naturally bioluminescent bacteria title was 10^{8} CFU/mL, and the assay procedures were done by mixing 500 µL of liquid cultures with 50 µL of heavy metals at 5,0 µg/mL or sanitizers, incubation during 1 h at 4º C, and the bioluminescence intensity of 90 µL of the treated suspension was measured in *cps* (counts per second) using an AB2200 luminometer (ATTO, Tokyo, Japan) during 10s.

Immobilized bacteria. The immobilization of the cells was made by mixing 1 mL of 1 mM IPTG induced cells at 10^8 CFU/mL and 9 mL of Top Agar (LB broth with 0.7% (w/v) agarose) with 1 mM IPTG at 42° C, and quickly transferring 30-100 µL of this mixture in each well of the 96-well microplate. The Elisa plate was stored at 4° C and bioluminescence was weekly analyzed in Light Capture CCD II Camera (ATTO, Tokyo, Japan) after addition of 10 µL of 10 mM D-luciferin at pH 5.0 to each well.

Results and Discussion

***In vivo* bioluminescence properties of beetle luciferases (Fig. 1).** In order to analyze the suitability of these beetle luciferases for *in vivo* biosensing, we first investigated the bioluminescence properties such as kinetics, spectra and activity of these luciferases inside the bacterial cells and compared with the *in vitro* properties of these luciferases already reported elsewhere [24, 25]. The luciferase of *Macrolampis* firefly displays an *in vitro* flash like kinetics, whereas *Pyrearinus* luciferase displays a slower kinetics. In living bacteria *Macrolampis* firefly luciferase bioluminescence also displays a fast decay with a half-life $t_{½}$ = 60 seconds, whereas *Pyrearinus termitilluminans* click beetle luciferase displays a more intense and sustained luminescence with a half-life $t_{½}$ = 330 seconds, lasting several minutes (Fig. 1A). Therefore, *Macrolampis* luciferase is more suitable for fast and more sensitive luminometric assay, whereas *P. termitilluminans* luciferase is more suitable for long term integration and less sensitive detection assays such as CCD and photographic analysis, due to its higher intensity and more sustained luminescence kinetics.

The luciferase of *Macrolampis* displays a broad spectrum with peak at 564 nm (Fig. 1B), whereas the luciferase of *Pyrearinus termitilluminans* displays blue-shifted spectrum with peak at 534 nm. The *in vivo* bioluminescence spectrum of *E. coli* expressing *Macrolampis* firefly luciferase is reddish in the beginning, gradually shifting toward the

yellow as time passes. This is a general property of firefly luciferases which are pH-sensitive, and may result from intracellular pH changes caused by providing luciferin in acidic buffer. Higher temperatures also tend to red-shift the spectrum of this luciferase. On the other hand, *Pyrearinus termitilluminans* emission spectrum is insensitive to pH, time and temperature.

Sensitivity of the bioluminescence assay (Fig. 2). The sensitivity of the bacterial *in vivo* detection assay was measured by CCD imaging and luminometry, by counting the minimal number of colony forming units (CFU/mL) able to produce a detectable signal using the method of microbiological assay describe in material and methods. By luminometry, up to 10 CFU/mL could be detected after 10s of signal integration. In CCD assay, up to100 CFU/mL could be detected by imaging, after 5 min of exposure at high sensitivity. In both CCD imaging and luminometric assays, the luciferase of *Pyrearinus termitilluminans* displayed the most intense and sustained luminescence, being potentially useful for less sensitive detection assays that require light integration, whereas the luciferase of *Macrolampis* sp due to its fast and slightly less intense signal could be useful for fast and more sensitive luminometric assays.

Effect of toxic agents on *in vivo* and *in vitro* bioluminescence (Fig. 3 and 4). In order to test the feasibility of using bacterial cells transformed with beetle luciferases for general toxicity biosensing, we analyzed the effect of heavy metals, anti-septic and sanitizers on *in vivo* bioluminescence, cell growth and mortality.

Heavy metals (Fig. 3). Among heavy metals, we tested copper, zinc, lead, mercury, silver and nickel at the final concentration of 5.0 μg/mL. We usually used the sulphate or chloride salts because their anions have little inhibitory effect on luciferase activity. Among them, $CuSO_4$ (0.02 mM), $ZnSO_4$ (0.0173 mM), $HgCl_2$ (0.0184 mM), $AgNO_3$ (0.0294 mM) and $NiSO_4$ (0.019 mM) showed the strongest inhibitory effects on bioluminescence intensity of live bacteria, except for $PbCl_2$ (0.0174 mM). When testing their effect on *in vitro* luciferase

reaction as a control for enzymatic inhibition, copper, zinc and nickel slightly inhibited luminescence intensity (60%, 50% and 40% respectively), indicating that at least part of the inhibitory effect on bacterial bioluminescence could be due to enzymatic inhibition and not necessarily metabolic inhibition or the toxic effect in the cells. The metals nickel, copper and mercury strongly inhibited the luciferase, probably by targeting sulphydryl or histidyl groups or by competing with the ATP on the reaction, decreasing the available MgATP and consequently the bioluminescence [29]. Analysis of cell growth inhibition and mortality assays showed that among these metals, copper, mercury and silver killed the bacteria. However, although mercury and silver at 5.0 μg/mL did not inhibited the enzyme, they strongly inhibited light emission, indicating that bioluminescence could be used as a good indicator of cell toxicity for these agents.

Then we analyzed the dose effect relationship of the metals which displayed highest inhibitory effect on *in vivo* bioluminescence assay, such as copper, zinc, mercury, silver and nickel. We analyzed the effect of these heavy metals at the concentration of 1, 2, 3, 4 and 5 μg/mL (Fig. 4).

We also observed growth inhibition of bioluminescent *E. coli* with zinc, cooper, mercury and silver at the final concentration of 0.5 μg/mL, which is similar to the concentration usually found in metal-contaminated rivers resulting from drainage of agricultural lands, where the metals are used in fertilizers and pesticides [30].

When compared with *Vibrio fischeri* cells from Biolux kit, the bioluminescence of bacteria transformed with beetle luciferases displayed higher sensitivity to silver and nickel, whereas cells from Biolux kit where more sensitive to zinc, copper and lead. The bioluminescence of both kind of cells were dramatically inhibited by mercury.

Altogether these results show that although bioluminescent *E. coli* transformed with beetle luciferases cDNAs are not good *light off* biosensor for most heavy metals, they are

quite sensitive to mercury and silver showing that bioluminescence inhibition reflects cell mortality in the latter.

Disinfectant and sanitizing reagents (Fig. 5). We also tested the effect of disinfectant agents commonly used in sanitization on *in vivo* (Fig. 5A) and *in vitro* (Fig. 5B) bioluminescence and on the cell mortality by the tetrazolium method (Fig. 5C). The following reagents were tested at the concentration of 5.0 μg/mL: cresol red (0.0244 mM), sodium carbonate (0.0471 mM), potassium dichromate (0.0169 mM) and 1-propanol (0.0831 mM). At these concentrations, most of the agents did not inhibit the *in vitro* bioluminescence of the luciferases of *Pyrearinus termitilluminans* and *Macrolampis*. However, *Macrolampi*s luciferase was inhibited by 1-propanol at 5.0 μg/mL (99%). Noteworthy, cresol red and potassium dichromate increased the luminescence activity of *Macrolampis* and *Pyrearinus* by 25% e 40%, respectively. Comparatively, in the *in vivo* luminometric assay using the *Vibrio fischeri* from Biolux kit, bacteria were more resistant to sodium carbonate than bioluminescent *E. coli* transformed with *Macrolampis* firefly luciferase (Fig. 5A).

We also tested the effect of the following sanitizers: Fenol 1%, Iodine 2%, Boric Acid 3%, Ethanol 70% and Hydrogen Peroxide 3% (these concentrations are the commonly used in antiseptic and bactericides formulations). Iodine inhibited both the growth (Fig. 5C) and the bioluminescence (Fig. 5D). In both figures 5A and 5B, we can detect the bioluminescence activity using the luminometer, and in the figures 5C and 5D we confirmed cell mortality using both the tetrazolium method and the bioluminescence activity in CCD Camera, where only iodine inhibited the microrganisms in both assays.

Immobilized bacteria (Fig. 6). In order to have a more stable assay format for field bioluminescence measurements, we have immobilized the bioluminescent bacteria in an agarose matrix in an Elisa plate, based on a previously established and patented application method [31, 32]. In this format, the immobilized bacteria stored at 4° C, kept their

bioluminescence activity constant during 1 week and up to 3 weeks with gradual decrease of bioluminescence activity. The bioluminescence was weekly imaged in Light Capture II CCD Camera (ATTO, Tokyo) after addition of 10 mM D-luciferin, in presence of $ZnSO_4$ 5.0 µg/mL to check cell mortality/inhibition of bioluminescence.

Similar results were recently obtained by Roda et al. [33], with whole cell biosensors (*Escherichia coli* JM109 strain expressing wild-type *Photinus pyralis* luciferase) immobilized in microplates, which kept their activity during one month when kept at 4° C in agarose, PVP and collagens. Park et al. [34] and Kim & Gu [35] also showed that bioluminescence of immobilized bacteria in microplates, remained stable during two weeks at 4° C, and gradually decreases every week until the fourth week. Comparing our immobilized bacteria transformed with pPro-Mac with those transformed with pPro-PY, the pPro-Mac showed higher bioluminescent signal and better stability.

Concluding remarks

Our studies with recombinant *E. coli* expressing two new beetle luciferases showed that both *Macrolampis* firefly and *Pyrearinus termitilluminans* luciferases are suitable for *light off* bacterial biosensors for general toxicity assays, showing detection sensitivities comparable or superior to naturally bioluminescent bacteria *Vibrio fischeri*. *Macrolampis* firefly luciferase displays lower bioluminescent signals and fast decay, being potentially useful for fast sensitive luminometric assays. The luciferase from *Pyrearinus termitilluminans*, due to its stronger and more sustained kinetics, appears to be more useful in less sensitive and inexpensive detection assays that require light integration for longer time, and which are not affected by intracellular variables such as pH, heavy metals and temperature. Although bioluminescent bacteria displayed a considerable sensitivity to mercury and silver correlating bioluminescence with cell mortality, they usually did not show specificity for any class of

toxicants, being therefore potentially useful only as general toxicity *light off* biosensors and also showing potential for bioprospection of anti-septic agents.

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Fig. 1. *In vivo* bioluminescence properties of beetle luciferases: (A) luminescence kinetics (the half-life of luminescence was $t_{½}$ = 60 s for *Macrolampis* and $t_{½}$ = 330 s for *Pyrearinus termitilluminans* luciferases); (B) bioluminescence spectra of *E. coli* expressing *Macrolampis* sp2 (black) and *Pyrearinus termitilluminans* (gray) luciferases; (C) bioluminescent activity *in vivo* and *in vitro*.

Fig. 2. Relationship between the number of bioluminescent *E. coli* expressing (A) *Macrolampis* sp2 and (B) *Pyrearinus termitilluminans* luciferases and bioluminescence intensity measured by luminometry with an ATTO AB2200 luminometer.

Fig. 3. Cell mortality and bioluminescence inhibition assays caused by heavy metals: (A) *E. coli in vivo* and (B) luciferase *in vitro* reaction with heavy metals at concentration 5.0 µg/mL.

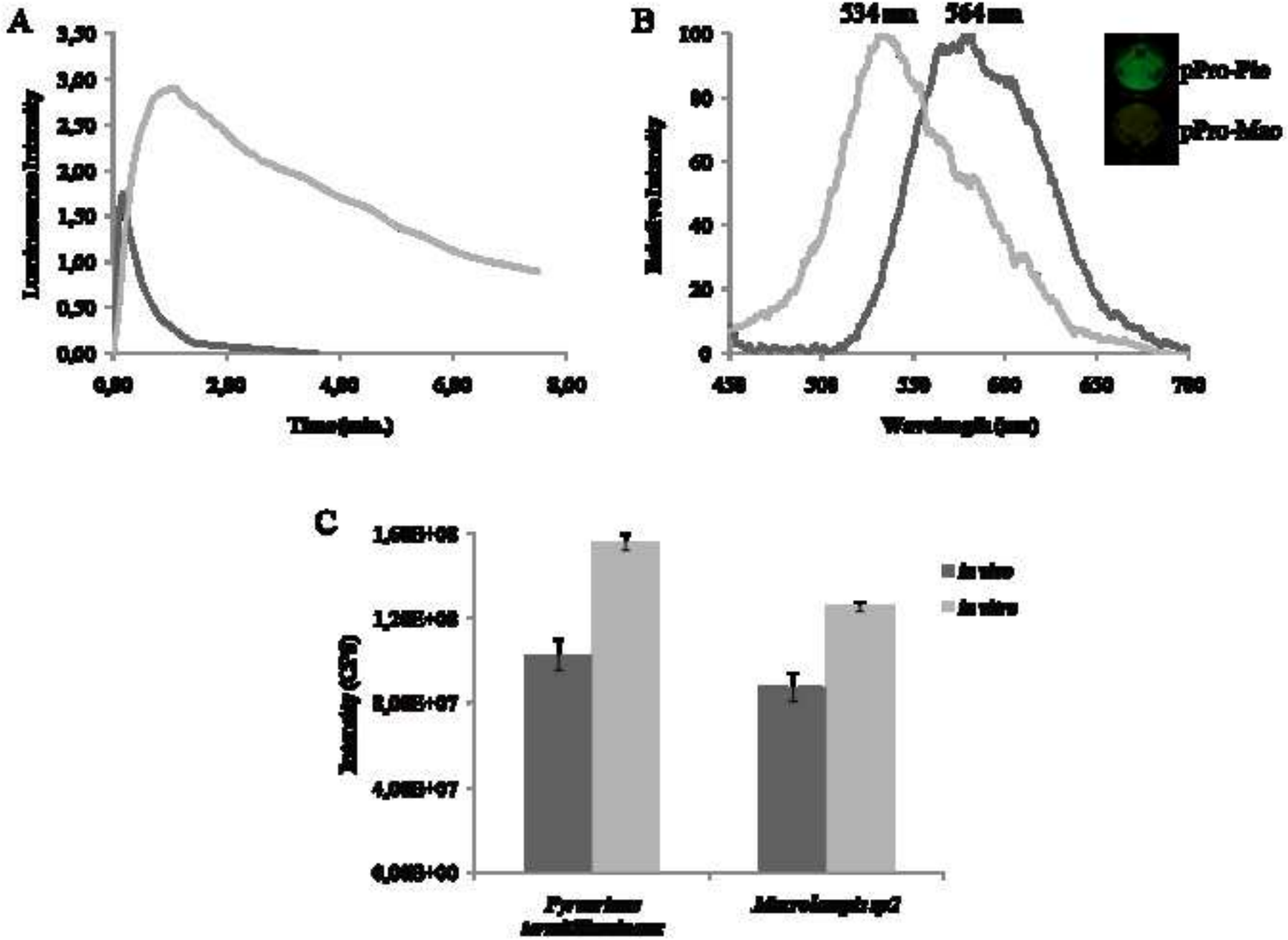
Fig. 4. Dose response curve on *in vivo* bioluminescence assay by heavy metals on *E. coli* expressing (A) *Macrolampis* sp2 and (B) *Pyrearinus termitilluminans* luciferases: (= control); (= $NiSO_4$); (= $CuSO_4$); (= $ZnSO_4$); (= $AgNO_3$) and (= $HgCl_2$).

Fig. 5. Effect of disinfectants on bioluminescence inhibition assay and cell growth/mortality: (A) *in vivo* (comparison of the bioluminescence inhibition assays using *E. coli* transformed with beetle luciferases and naturally bioluminescent bacteria *Vibrio fischeri* from Biolux kit)*;* (B) effect on *in vitro* bioluminescence with potassium dichromate ($K_2Cr_2O_7$), sodium carbonate (Na_2CO_3), cresol red (CH_4OH) and 1-propanol (C_3H_8O) at concentration 5.0 µg/mL and 3% fenol (C_6H_5OH), 2% iodine (I), 3% boric acid (H_3BO_3) 70% ethanol (EtOH) and 3% hydrogen peroxide (H_2O_2); (C) tetrazolium colorimetric assay and (D) CCD

bioluminescence imaging of *E. coli* transformed with pPro-Mac and pPte-PY with sanitizing agents 1% fenol [C_6H_5OH], 2% iodine [I], 3% boric acid [H_3BO_3], 70% ethanol [EtOH] and 3% hydrogen peroxide [H_2O_2] after incubation during 1 hour at 4° C.

Fig. 6. Bioluminescence of immobilized *E. coli* in agarose gels during four weeks storage at 4° C and assay with 5 µg/mL $ZnSO_4$.

Figure



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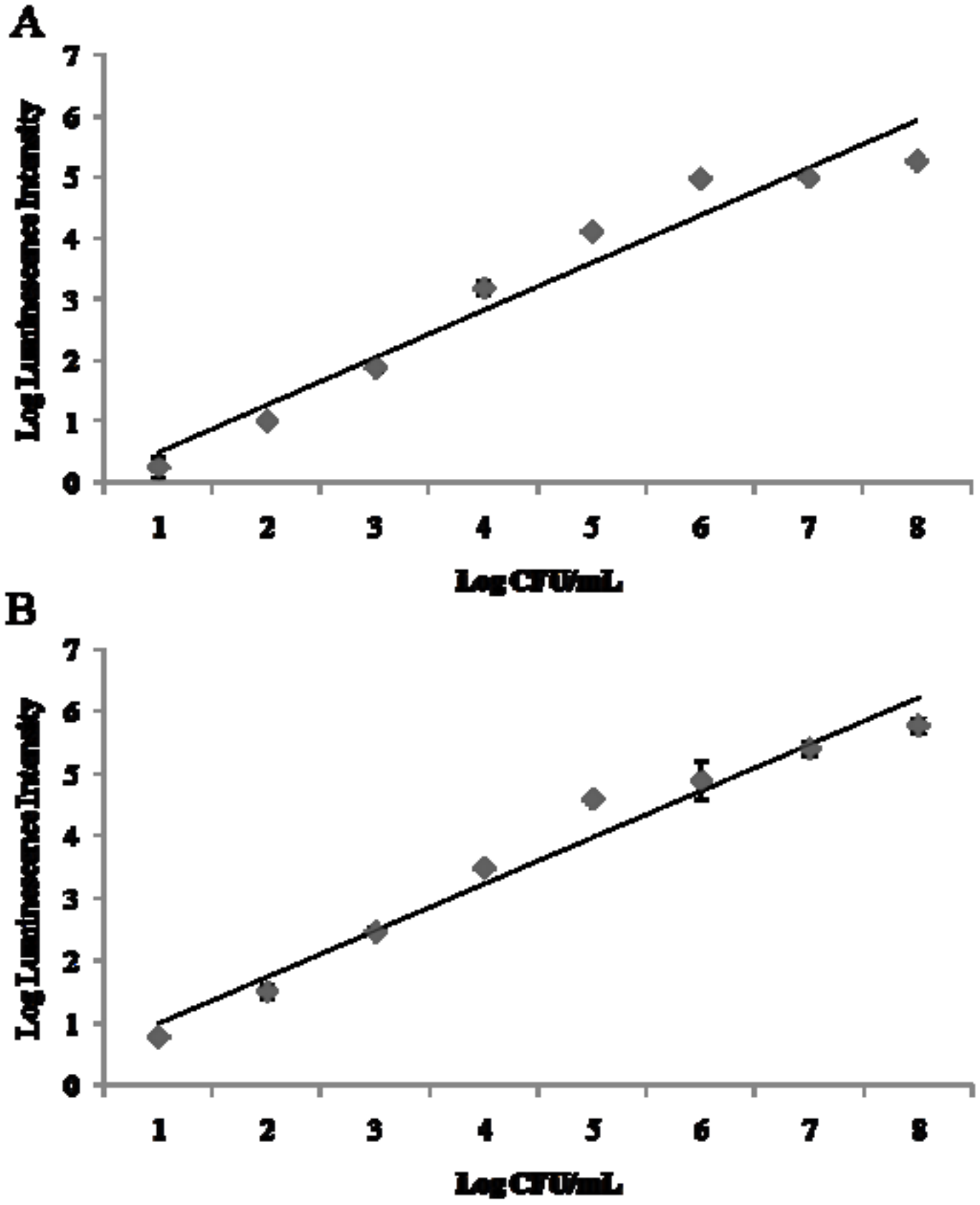

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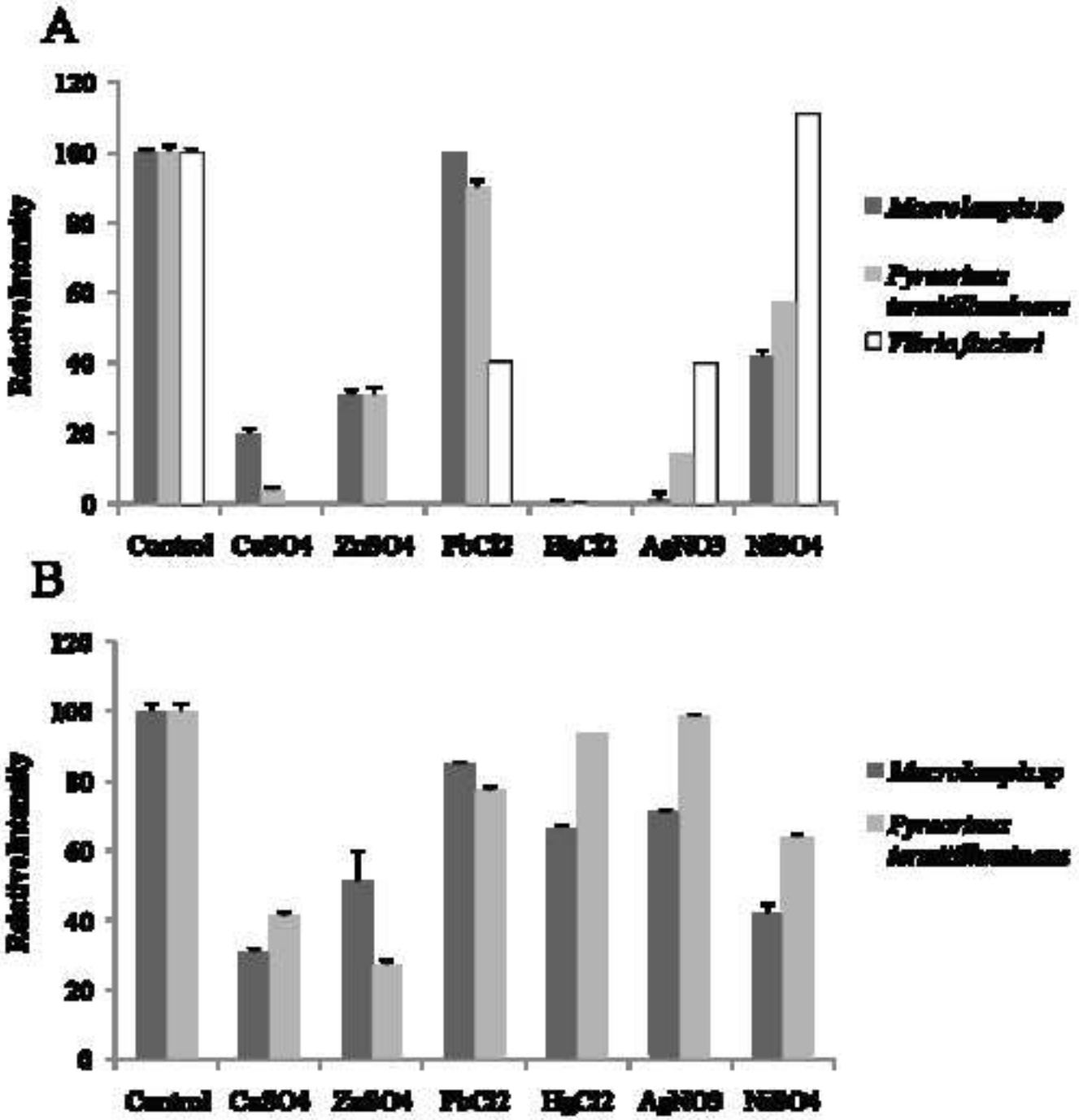

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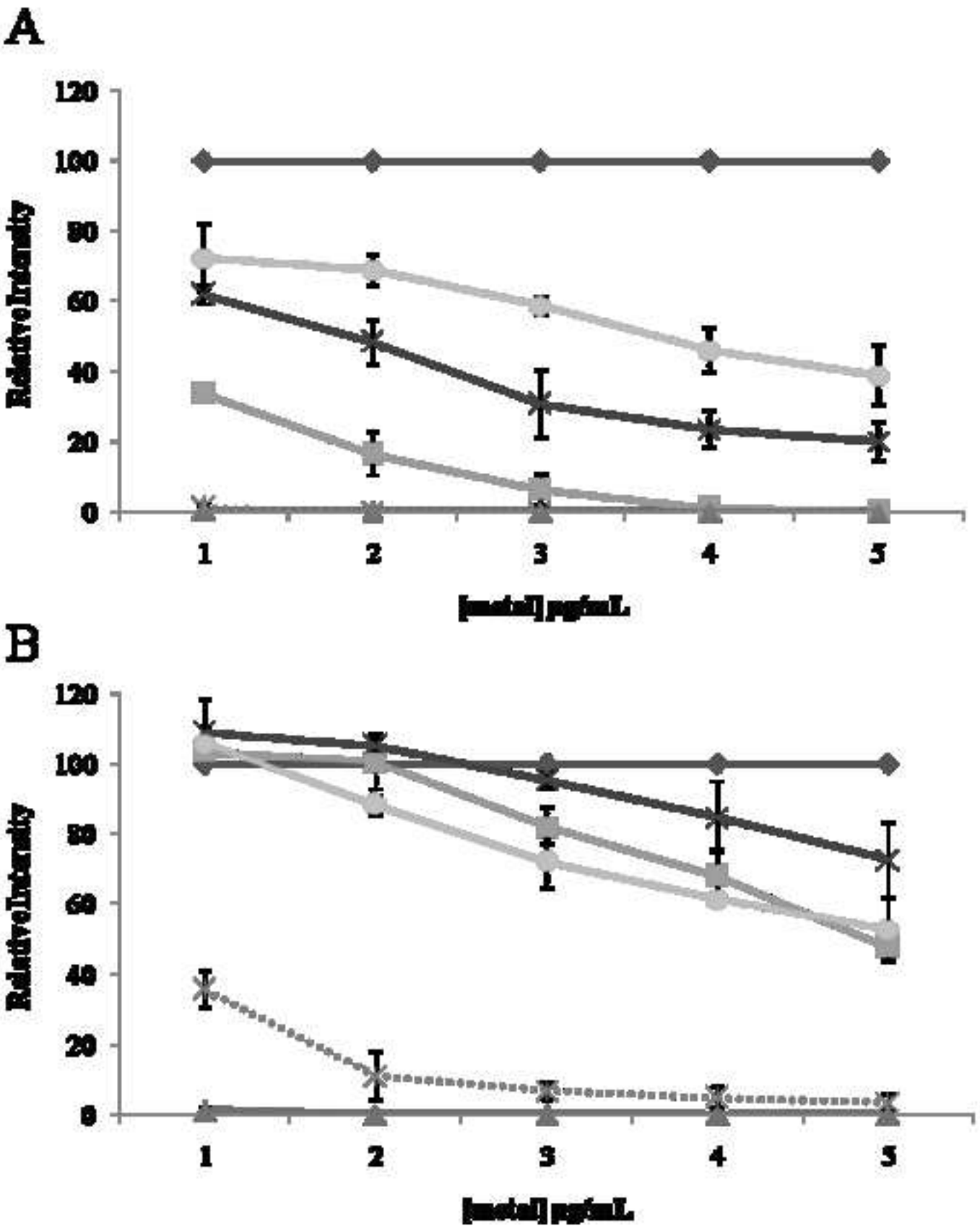

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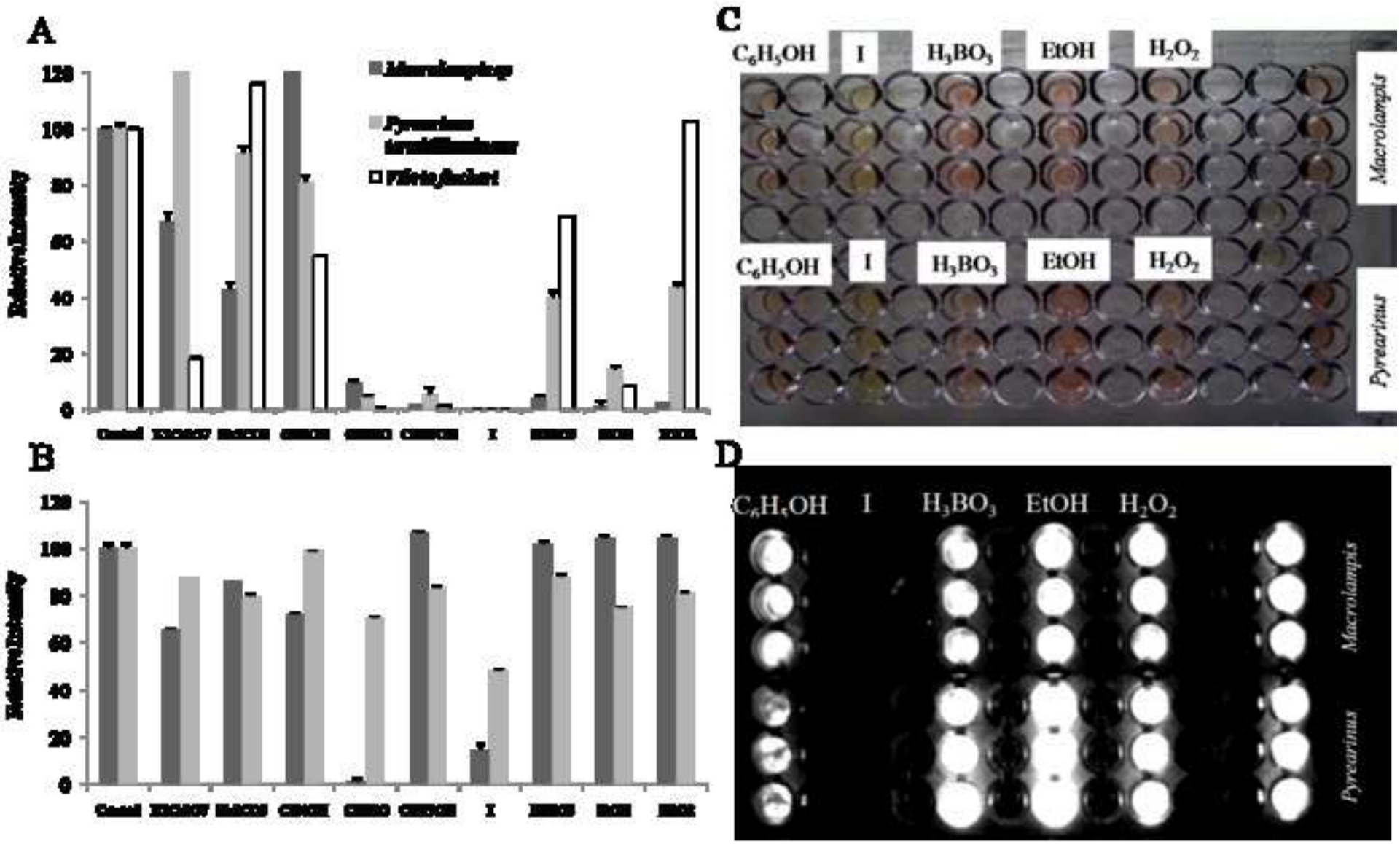

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