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APPLICATION OF A KIT-FREE ECOTOXICITY ASSAY BASED ON THE LUMINESCENCE OF *VIBRIO FISCHERI* IN THE TESTING OF SUNSCREENS

Pierinna M. Ratto[a,b], Lisveth V. Flores del Pino[b,c], Ana A. Kitazono[a,d]

ABSTRACT

One of the most used ecotoxicity bioassays is based on the luminescence of *Vibrio fischeri*, for which commercial kits such as Microtox® and several others are available. However, this assay might not always be suitable for several reasons: Its protocol cannot be easily adapted for particular purposes, and it requires the continuous purchases of the kit and the availability of specialized equipment or at least, a luminometer. As a versatile alternative, this paper describes an optimized and kit-free protocol, referred to as "Macrotox", which allows a wide range of treatment periods (for example, 1, 3, 7, 12, 24, 30 and 36 h) to follow the effects on the luminescence and proliferation of *V. fischeri*. While the optimized protocol involves 1-mL treatments, it could be easily adapted to use other volumes and times. Importantly, to evaluate the effects on luminescence, only a digital camera is needed to take photographs in the dark, which are then analyzed using the open access software ImageJ. To evaluate the effects on proliferation, cell densities are estimated via spectrophotometric measurements. In this work, both the Microtox® and Macrotox protocols were applied for the determination of the ecotoxicities of a commercial sunscreen and a moisturizer as a control. However, the described Macrotox protocol can be similarly applied for the ecotoxicity assay of any sample.

Key words: Microtox®; ecotoxicity of sunscreens; *Photobacterium phosphoreum*; *Allivibrio fischeri*.

[a] Laboratory of Biological Chemistry and Bioanalysis, Academic Department of Chemistry, School of Sciences, Universidad Nacional Agraria La Molina, Perú.
[b] Master Program in Environmental Sciences, Universidad Nacional Agraria La Molina, Perú.
[c] Center for Research in Chemistry, Toxicology and Environmental Biotechnology, Universidad Nacional Agraria La Molina, Perú.
[d] Corresponding author. Academic Department of Chemistry, School of Scienes, Universidad Nacional Agraria La Molina - Av. La Molina s/n - La Molina - Lima – Perú - anakitazono@lamolina.edu.pe

APLICACIÓN DE UN ENSAYO ECOTOXICÓGICO QUE NO REQUIERE EL USO DE UN KIT Y SE BASA EN LA LUMINISCENCIA DE *VIBRIO FISCHERI* PARA EL ANÁLISIS DE BLOQUEADORES SOLARES

RESUMEN

Uno de los más usados bioensayos de ecotoxicidad se basa en la luminiscencia de *Vibrio fischeri*, para el que existen kits comerciales como el de Microtox® y varios otros. Sin embargo, este ensayo no puede ser siempre aplicado por varias razones. Por un lado, su protocolo no puede ser fácilmente adaptado o modificado, y además, requiere la adquisición continua del kit y la disponibilidad de equipamiento especializado o por lo menos, de un luminómetro. Como una alternativa versátil, este artículo describe un protocolo optimizado que no requiere el uso de un kit, denotado como "Macrotox", que permite un rango amplio de periodos de tratamiento (por ejemplo, 1, 3, 7, 12, 24, 30 y 36 h), siguiendo los efectos en la luminiscencia y proliferación de. *V. fischeri*. Aunque el protocolo optimizado envuelve tratamientos de 1 mL, puede ser fácilmente adaptado para usar otros volúmenes y tiempos. Es importante destacar que, para evaluar los efectos en la luminiscencia, solo una cámara digital es necesaria para tomar las fotografías en la oscuridad, las que son luego analizadas mediante el software de acceso abierto Image J. Para la evaluación de los efectos sobre la proliferación, las densidades celulares son estimadas mediante mediciones espectrofotométricas. En este artículo, ambos métodos Microtox® y Macrotox fueron aplicados para la determinación de los niveles de ecotoxicidad de un bloqueador comercial y una loción humectante como control. Sin embargo, el protocolo Macrotox descrito puede ser similarmente aplicado para el ensayo ecotoxicológico de cualquier otra muestra.

Palabras clave: Microtox®; ecotoxicidad de fotoprotectores, *Photobacterium phosphoreum*; *Allivibrio fischeri*.

INTRODUCTION

The quality of water and other environmental matrices is usually investigated via independent assays that determine several physicochemical and biological parameters[1]. However, this determination is not fully accurate because the resulting data only represent the quality of the sample at a defined moment in time. On the other hand, it is possible to rely on the Water Quality Indexes (WQI), which are based on mathematical calculations that involve the values of the parameters established in the Environmental Quality Standards. However, it is important to consider that obtaining results that are within the WQI cannot always be taken as a guarantee for the integrity of an ecosystem. Ecotoxicity assays arise from the need to evaluate and prevent the ecological damage that could be caused by the presence of chemical or other agents in a natural environment[2]. These tests, commonly called bioassays, use biological units to evaluate the toxic effect of a contaminated environmental matrix on individual characteristics such as survival, development, reproduction, etc.[3]. There is a wide range of methodologies using various

organisms to assess toxicity of aquatic ecosystems[4,5]. Among the best known assays are those that evaluate the effects on the mortality and reproduction of the planktonic crustacean *Daphnia magna*[1], and the germination and radicle growth of the seeds of the lettuce *Lactuca sativa*[6].

However, because of its simplicity, one of the most frequently used assays is based on the luminescence of the marine bacterium *Vibrio fischeri*[7]. This bacterium emits a blue-green light through a reaction catalyzed by the luciferase enzyme between the reduced flavin mononucleotide (FMNH2), an aldehyde compound and oxygen, to produce oxidized flavin mononucleotide (FMN), water, and a carboxylic acid [8]. This reaction is linked to cellular respiration through the electron chain and, therefore, any compound that affects these processes will cause a decrease in luminescence levels[9].

The ecotoxicity assay based on the luminescence of *V. fischeri* was developed by the Beckman Instrument Co. in 1979 as a faster and simpler procedure than the traditional fish and invertebrate tests[10]. Over time, the methodology was validated and approved by several international organizations, and the first standardized protocol was implemented in 1984 by the Organization for Economic Cooperation and Development (OECD)[10]. Subsequently, different organizations adopted and updated the same methodology, implementing for example, the ISO 11348-3:2007, "Determination of the inhibitory effect of water samples on the luminescence of *Vibrio fischeri* (luminescent bacterial assay)". Since that time, several companies started commercializing kits applying this methodology (Microtox, LumiStox, ToxAlert, Biotox, BioLight, BioFix, and others), which include the required amounts of a lyophilized culture and solutions, and a detailed protocol[11,13].

The ecotoxicity assay kits based on *V. fischeri*, generalized as Microtox®, have become very popular because they can be used with various types of samples, are very sensitive, and the results are obtained in a short time[9]. Despite the advantages of performing this standardized bioassay, several investigators have reported problems involving the inability to detect long-term effects, not only on luminescence emission, but also on cell proliferation and other physiological processes[14,15]. Another disadvantage is the need to purchase the kit every time the assay needs to be performed, since the provided lyophilized *V. fischeri* culture needs to be used immediately after it is reconstituted. Additionally, an important aspect to consider is the initial investment cost for the acquisition of the equipment required for the luminescence measurements: A luminometer, fluorescence spectrophotometer, or a more specialized equipment.

In this study, the objective was to optimize a kit-free ecotoxicity assay, which was based on the luminescence of *V. fischeri* and denoted as "Macrotox". As a proof of principle, this assay was applied in the testing of sunscreens and the results were compared to those obtained with the classical Microtox kit.

EXPERIMENTAL PART

Culture media

V. fischeri was grown and treated using the "Luria-Bertani salt" (LBS) medium and an adaptation of the "sea water medium" (SWM), respectively (16, 17). The first, used to grow *V. fischeri*, was composed of 10 g/L tryptone, 5 g/L yeast extract, 20 g/L NaCl and 0.05 M Tris buffer pH 7.5. The second, used in the bioassays, contained 28.1 g/L NaCl, 0.8 g/L KCl, 1.6 g/L $CaCl_2.2H_2O$, 3.5 g/L $MgSO_4.7H_2O$ and 10 g/L peptone.

Equipment

For the sample preparations, a refrigerated centrifuge (ThermoScientific Sorval Legend X1R) and a microcentrifuge (ThermoScientific MicroCL17) were used. The luminometer used for the Microtox® assays (Berthold Detection System GmbH) was kindly provided by Dr. Javier Quino (University of Lima). The proliferation assay was based on absorbance determinations using a ThermoScientific Genesys 10S spectrophotometer. Macrotox", while a digital camera (Canon EOS 1200D) was used to determine the effects on luminescence.

Sample preparation

The analyzed commercial sunscreen included as photoprotective actives homosalate, octocrylene, avobenzone, octisalate, bemotrizinol, titanium dioxide (nano) and ensulizole. The extraction of the components was performed using dimethyl sulfoxide (DMSO) and distilled water, following the steps described in figure 1[4,13,18].

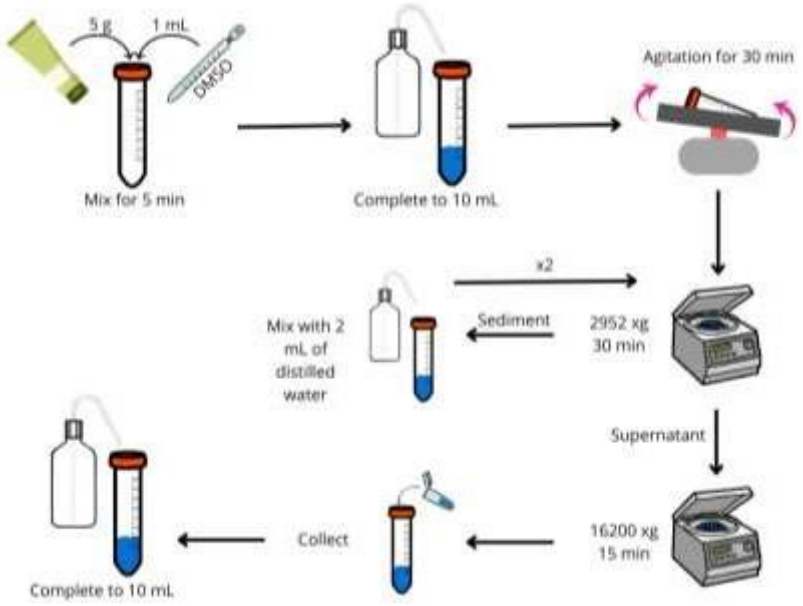

Figure 1: Representation of the procedure followed for sample preparation. Five grams of the sunscreen or moisturizer were weighed and mixed with 1 mL of DMSO for 5 min. Then, water was added to complete a final volume of 10 mL and the resulting mix was homogenized first manually and then by means of a rotary shaker for 30 min. The tubes were centrifuged and the collected supernatants were centrifuged again to remove any remaining solid particles. On the other side, the sediment from the first centrifugation was mixed with 2 mL of water for another round of extraction, and these cycles were repeated two more times. The supernatants were collected in a single container and water was added to a final volume of 10 mL. Therefore, each of these solutions includes all the material extracted from 5 g of sample (sunscreen or moisturizer).

Ecotoxicity assay using the Microtox® system

The BioTox™ WaterTox™ EVO commercial kit (Environmental Bio-Detection Products Inc. (Canada) was used, which applies the conventional Microtox method (ISO 11348- 3:2007). Measurements were performed using a Berthold Detection System

GmbH luminometer, which was kindly provided by Dr. Javier Quino-Favero of the Research Group on Technological Solutions for the Environment of the Universidad de Lima (Peru).
V. fischeri suspensions were treated with 1% DMSO (control), 50 g/L of moisturizer, or five different concentrations of sunscreen (3.1, 6.3, 12.5, 25 and 50 g/L). These samples were prepared using 2% NaCl, following the supplier's instructions. Measurements were carried out at the beginning of the treatment and after 15 minutes. The calculation of the percentage inhibition was made by applying the following equation:

$$Inhibición\ (\%) = 100 - 100 * \left(\frac{L_{15}}{L_0 * k_f}\right)$$

Where L_0 and L_{15} represent the luminescence read in RLU (Relative Light Unit) at times 0 and 15 minutes, respectively; while k_f is a constant obtained by dividing the luminescence of the control (1% DMSO) at time 15 over that obtained at time 0 [19].

"Macrotox" ecotoxicity assay

The *V. fischeri* strain used in these assays was recovered from the purchased kit (BioTox™ WaterTox™ EVO). These cells were grown in LBS medium under constant agitation at 25 ºC for up to 16 hours. The cell suspensions were diluted to OD_{600nm} of 0.2 using SWM medium, always maintaining an LBS:SWM media ratio of 1:2 to ensure that all assays were performed at the same nutrient concentration. The prepared extracts (moisturizer, sunscreen) or DMSO were included in the cell suspensions to start thetreatments as follows: One control with 1% DMSO, another with 50 g/L of the moisturizer, and different concentrations of the sunscreen (see figure 2). The samples were distributed in 24-well plates (one for each time point), transferring 1 mL and considering three replicates. The plates were kept in a water bath at 25 ºC with constant agitation for the required time[17].
The treatments were run for 1, 3, 7, 12, 24, 30 and 36 h, thus requiring seven 24-well plates. For the luminescence assays, photos of the plates were taken in the dark, adjusting the parameters of a Canon EOS 1200D camera to a sensitivity of ISO 6400 and an exposure time of 8 seconds. Each of the images obtained was processed with the well-known public domain software ImageJ (https://imagej.net/ij/download.html), generating a circular area of common size for all the wells of the plates. The program was configured to measure, based on the plotted area, the gray value average, calculated from the average value of the intensity of the RGB bands of each image. On the other hand, to determine the effect on cell proliferation, spectrophotometric readings were taken at 600nm, immediately after capturing the luminescence image. All tests were repeated twice, on different dates.

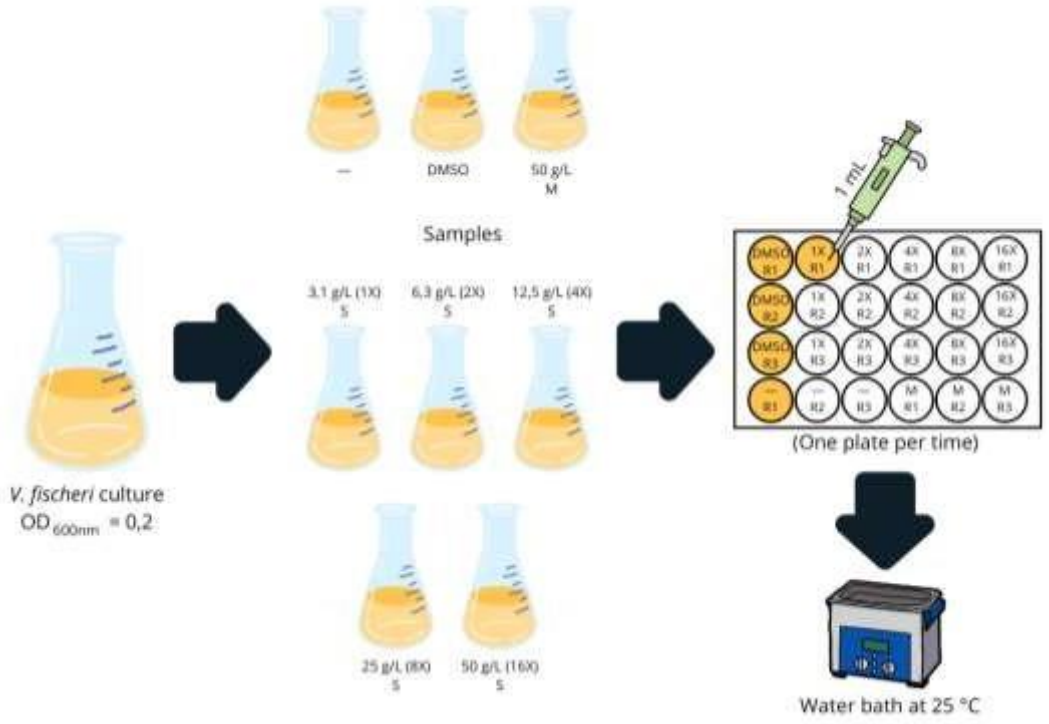

Figure 2: Optimized protocol for the "Macrotox" assay. With the *V. fischeri* culture at the desired cell density, the respective treatment samples and controls were prepared as indicated: "–", a control without any addition, "DMSO" a control with 1% DMSO, "M" a sample control that contained 50 g/L of the moisturizer; and five sunscreen samples with different concentrations, from 3.1 g/L (1X) to 50 g/L (16X). Then, 1 mL of each suspension was transferred, in triplicate, to 24-well plates (one for each time point), which were placed in a water bath at 25 °C under constant agitation for the indicated times.

RESULTS AND DISCUSSION

As indicated above, although the Microtox® method is widely applied, it has important disadvantages and among these, a very important one is related to its cost and the requirement of specialized equipment or at least, a luminometer. Additionally, the possibilities of applying the assay are limited to the availability of the lyophilized cells (purchase of the kit) and to the established protocol. The main objective of this work was to optimize a kit-free, in-house protocol, applying it for the determination of the ecotoxicity of a sunscreen and a photoprotective active ingredient. Ecotoxicity studies were performed with samples prepared similarly, using the standard assay with a commercial kit (Microtox®) and with a more conventional kit-free protocol based on cultures of *V. fischeri* denoted as "Macrotox".

The results of the Microtox® assay are listed in table 1. When evaluating the results of the sunscreen, an increase in luminescence is seen in the treatments with concentrations equal to or lower than 12.5 g/L, while the samples with 25 and 50 g/L causes the opposite effect, with the maximum inhibition levels of around 15.70%. In turn, the moisturizer showed 16.35% inhibition, a result that, surprisingly, was close to the inhibitory effect of the 50 g/L sunscreen sample (15.70%). It should be noted that cosmetic creams commonly include parabens, which can be toxic at very low concentrations to *V. fischeri*

(20). For this reason, it was important to include the moisturizer as a control in this assay and thus, it was expected to find stronger inhibitory effects in the presence of the photoprotective actives. Notably, these were not evident under the conditions of this assay, even at the highest concentrations of the sunscreen.

Table 1. Results of percentage inhibition of luminescence using the Microtox® assay

Sample		Inhibition (%)			Average	Standard deviation
		R1	R2	R3		
Moisturizer	50 g/L	20.17	13.82	15.09	16.36	3.36
Sunscreen	3.1 g/L	1.06	-1.95	-7.35	-2.74	4.26
	6.3 g/L	-3.91	-3.78	-8.47	-5.39	2.67
	12.5 g/L	1.72	-2.38	-4.79	-1.82	3.29
	25 g/L	12.17	6.62	2.95	7.24	4.64
	50 g/L	16.38	17.2	13.52	15.7	1.93

R1, R2, R3: Repetitions

On the other hand, the Macrotox assay was also performed following the methodology described in figure 2. For the luminescence assays, photos were taken of the plates containing the samples and the gray value of each one was determined using the ImageJ software. Figure 3 includes in the top panel a representative example of the photos taken in the dark (7-hour treatment), and at the bottom, a graph that includes the gray values obtained for all samples and treatments.

As shown in figure 3B, the luminescence levels increase in all samples during the first 12 hours of the test, decreasing with the passage of time until reaching values close to zero. However, even from the first time-point (1-hour treatment), the stronger inhibitory effect of the sunscreen is evident in a dose-dependent manner. During almost all the time, the 1% DMSO control is the one that achieved the maximum light emission, which can be noticed especially at 12 hours with a gray value above 80. This result is very different from the control that did not include the solvent ("–"), which showed luminescence levels up to a gray value of around 50. According to several reports, DMSO, at low concentrations, causes an inductive effect on luminescence, which could be explained by its ability to donate electrons, thus positively interfering with luminescence emission (Nasuhoglu *et al*., 2017).

Taking as a reference the results with the 1% DMSO control, the luminescence levels for the sunscreen samples decreased as its concentration increased, obtaining a gray value of approximately 40 at 12 hours in the 50 g/L treatment (16X), representing an inhibition of 53%. However, the visual differences are more noticeable at 7 hours, as can be seen in figure 3A, since the inhibition at that time reached a value close to 69%. As can be observed, higher concentrations of the sunscreen led to stronger inhibition of the luminescence of *V. fischeri*. Additionally, the moisturizer produced lower luminescence than the 8X sunscreen sample (25 g/L). Since the only difference between the sunscreen and the moisturizer is the presence of the photoprotective actives in the former, it could be inferred that the luminescence inhibition is due to these compounds.

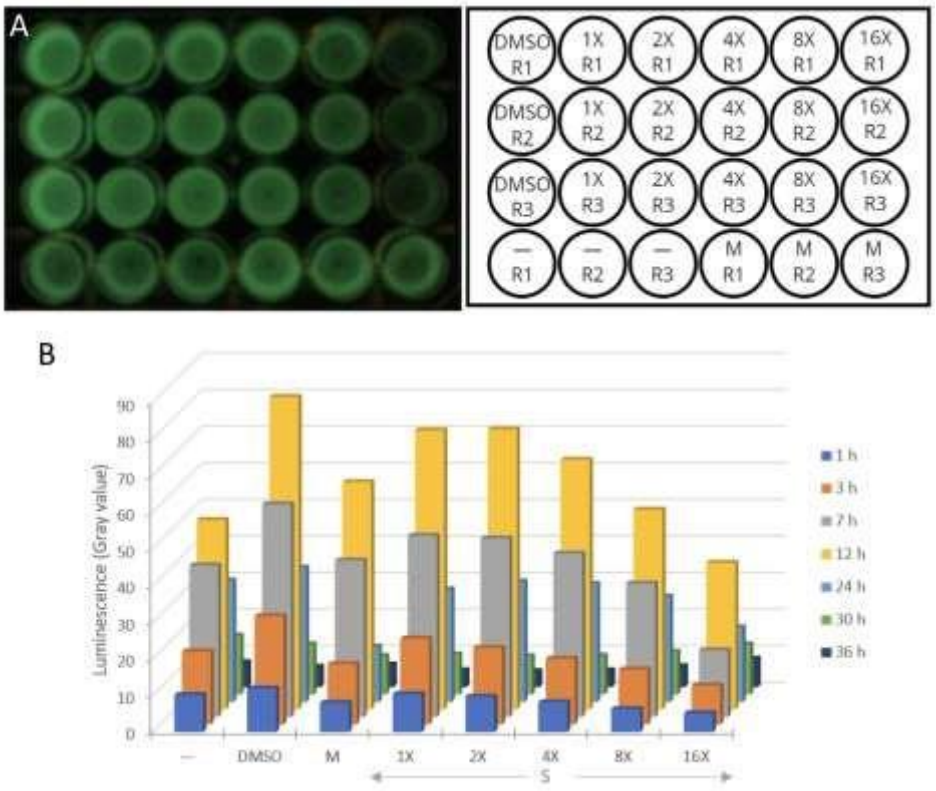

Figure 3: Representative results of the luminescence assays using the Macrotox protocol. A. A representative example of a photo taken from a plate that includes the controls and samples with the respective replicates (three in each case) after a 7-hour treatment. "–" is the control that includes only the V. fischeri culture, "DMSO" is the control with 1% of the solvent, "M" is the sample control with 50 g/L of the moisturizer, and the sunscreen treatments are denoted by the respective concentrations, from 3.125 g/L (1X) to 50 g/L (16X). **B.** The graph includes the mean gray values for each of the treatments, using the same nomenclature as in A. The numerical values of the results included in this figure are listed in Supplementary material 1.

Thus, the results presented so far show that, while using similarly prepared samples and controls, only the Macrotox protocol allowed the detection of the inhibitory effects on luminescence. Although no difference was detected between the moisturizer and the sunscreen using the Microtox® protocol (Table 1), the differences were obvious with the Macrotox assay even after a 1-hour treatment. Similarly, several Microtox® studies on UV filters have reported low toxicity with most of the compounds present in the sunscreen used in this study. One interesting exception is avobenzone, which shows luminescence inhibition levels above 50% [13].

Simultaneously, the proliferation assay was carried out with the sunscreen samples (figure 4). An increasing trend in cell density was observed until 24 - 30 hours, when the maximum level was reached in each case. In contrast to luminescence, it is observed that the inclusion of 1% DMSO causes a decrease in the levels of cell proliferation. As for the sunscreen samples, cell proliferation levels decreased as the concentration increased, with lower values than the controls. However, when compared with the DMSO control,

a similar toxicity was observed for the 4X and 8X sunscreen samples (12.5 and 25 g/L), while the moisturizer had a minor effect.

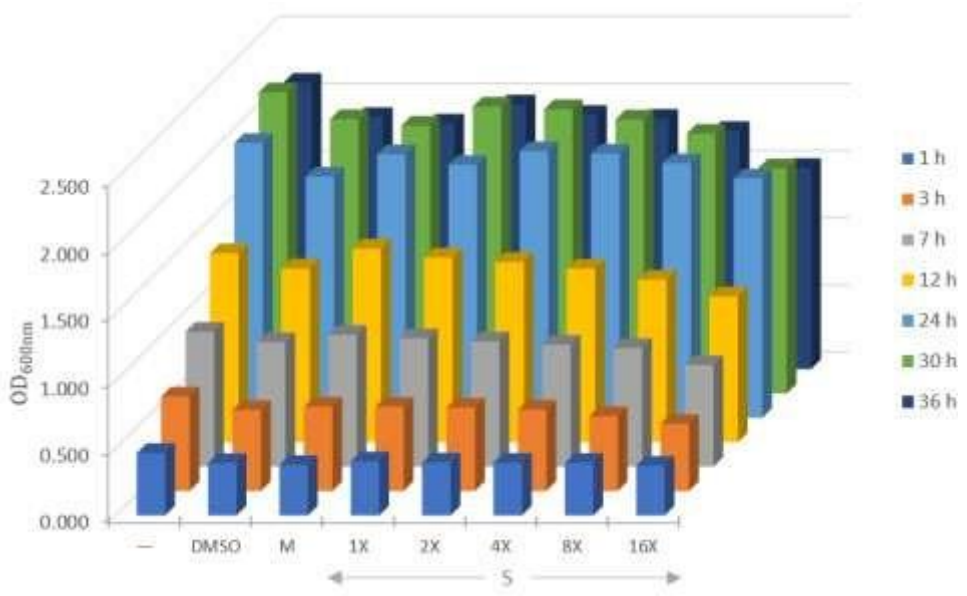

Figure 4. Results of proliferation assays using the Macrotox protocol. The OD600nm readings were performed on the controls and samples treated for the indicated times: "–" is the control that includes only the V. fischeri culture, "DMSO" is the control with 1% of the solvent, "M" is the sample control with 50 g/L of the moisturizer, and the sunscreen treatments are denoted by the respective concentrations, from 3.125 g/L (1X) to 50 g/L (16X). The numerical values of the results included in this figure are listed in Supplementary material 2.

A possible explanation for the inhibitory effect of the sunscreen could be the indirect action of the titanium dioxide nanoparticles. These are too large to pass through the bacterial cell membrane, but it can be adsorbed on the surface causing an increase in permeability, reducing fluidity, and promoting lipid peroxidation when the particles are positively charged[21]. These results demonstrate that the proliferation assay is a valuable complementary method for the evaluation of toxic effects, which importantly, as described in the Macrotox protocol, can be run simultaneously with the one for luminescence. It is noteworthy that no such study has been found for photoprotective actives, and only a few for other compounds. For example, Fulladosa *et al*.[22] reported the effects of organic arsenical compounds on the proliferation of *V. fischeri*. Accordingly, it is hoped that, the reported results and Macrotox protocol will facilitate both luminescence and cell proliferation studies.

CONCLUSIONS

The Macrotox protocol applied in this work allows the detection of toxic components in aqueous samples, especially when examining the effect on luminescence. Advantageously, besides its simplicity and lower cost, it can be easily adapted to run assays for 36 hours or more using the preferred sample volumes. This could be

demonstrated with the reported results, since, for example, the Microtox® protocol was not able to detect any effect of the sunscreen extracts on luminescence. On the other hand, the versatility of the Macrotox protocol allowed determination of 69% inhibition with 7-hour treatments.

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SUPPLEMENTARY MATERIAL

Supplementary material 1

Results of the Macrotox luminescence tests with samples containing sunscreen and moisturizer: Gray values were determined using the ImageJ software.

Sample	Result	1 h	3 h	7 h	12 h	24 h	30 h	36 h
—	**Average**	10.16	20.00	41.49	51.86	33.26	16.16	6.89
	SD	1.87	4.73	4.37	18.34	5.17	4.08	0.72
DMSO	**Average**	11.93	29.70	58.17	85.51	36.79	13.80	5.60
	SD	1.82	1.90	1.16	1.76	5.34	0.76	0.38
50 g/L Moisturizer	**Average**	8.00	16.55	42.84	62.19	15.27	10.60	6.23
	SD	1.92	1.19	2.49	7.66	5.73	1.81	2.21
3.1 g/L (1X) Sunscreen	**Average**	10.38	23.52	49.58	76.34	30.87	11.05	4.69
	SD	2.14	1.09	1.57	5.99	6.37	1.13	0.21
6.3 g/L (2X) Sunscreen	**Average**	9.69	20.96	48.86	76.62	32.99	10.53	4.46
	SD	2.22	0.58	1.30	2.38	2.56	1.53	0.31
12.5 g/L (4X) Sunscreen	**Average**	8.17	18.01	44.72	68.32	32.37	10.68	4.66
	SD	2.29	0.68	1.20	2.52	1.57	1.80	0.33
25 g/L (8X) Sunscreen	**Average**	6.30	15.06	36.54	54.68	28.94	11.74	5.80
	SD	1.97	0.91	2.01	6.29	2.37	1.76	0.45
50 g/L (16X) Sunscreen	**Average**	5.17	10.68	18.28	40.17	20.45	13.73	7.83
	SD	1.98	1.45	2.14	4.74	5.86	1.10	0.80

SD, standard deviation

Supplementary material 2

Results of the Macrotox proliferation tests (OD_{600nm}) with the samples containing sunscreen and moisturizer.

Sample	Result	1 h	3 h	7 h	12 h	24 h	30 h	36 h
—	**Average**	0.465	0.705	1.005	1.407	2.046	2.243	2.132
	SD	0.061	0.092	0.042	0.162	0.219	0.048	0.153
DMSO	**Average**	0.390	0.604	0.927	1.294	1.796	2.043	1.877
	SD	0.028	0.026	0.035	0.146	0.205	0.113	0.199
50 g/L Moisturizer	**Average**	0.376	0.628	0.982	1.442	1.963	1.995	1.828
	SD	0.032	0.030	0.057	0.158	0.084	0.111	0.158
3.1 g/L (1X) Sunscreen	**Average**	0.403	0.627	0.956	1.375	1.883	2.136	1.971
	SD	0.034	0.036	0.066	0.215	0.126	0.122	0.138
6.3 g/L (2X) Sunscreen	**Average**	0.394	0.622	0.933	1.342	1.986	2.117	1.893
	SD	0.028	0.032	0.079	0.196	0.059	0.170	0.128
12.5 g/L (4X) Sunscreen	**Average**	0.392	0.606	0.909	1.293	1.967	2.041	1.864
	SD	0.037	0.033	0.069	0.162	0.125	0.084	0.158
25 g/L (8X) Sunscreen	**Average**	0.392	0.558	0.884	1.211	1.896	1.935	1.773
	SD	0.030	0.010	0.065	0.134	0.147	0.037	0.091
50 g/L (16X) Sunscreen	**Average**	0.370	0.500	0.756	1.085	1.783	1.676	1.498
	SD	0.022	0.017	0.053	0.113	0.220	0.030	0.104

SD, standard deviation