Andrade, GC, Brancini, GTP, Abe, FR, de Oliveira, DP, Nicolella, HD, Tavares, DC, Micas, AFD, Savazzi, EA, Silva-Junior, GJ, Wainwright, M and Braga, GÚL

Phenothiazinium dyes for photodynamic treatment present lower environmental risk compared to a formulation of trifloxystrobin and tebuconazole

Phenothiazinium dyes for photodynamic treatment present lower environmental risk compared to a formulation of trifloxystrobin and tebuconazole

Gabriela Carvalho Andrade[1a], Guilherme Thomaz Pereira Brancini[1a], Flávia Renata Abe[1], Danielle Palma de Oliveira[1,2], Heloiza Diniz Nicolella[3], Denise Crispim Tavares[3], André Fernando Ditondo Micas[4], Eduardo Angelino Savazzi[4], Geraldo José Silva-Junior[5], Mark Wainwright[6], Gilberto Úbida Leite Braga[1*]

[1]University of São Paulo (USP), School of Pharmaceutical Sciences of Ribeirão Preto, 14040-903 Ribeirão Preto, SP, Brazil

[2]National Institute for Alternative Technologies of Detection, Toxicological Evaluation and Removal of Micropollutants and Radioactivies (INCT-DATREM), São Paulo State University (UNESP), Institute of Chemistry, 14800-060, Araraquara, SP, Brazil

[3]University of Franca, Laboratory of Mutagenesis, 14404-600, Franca, SP, Brazil

[4]Companhia Ambiental do Estado de São Paulo (CETESB), Divisão de Laboratório de Ribeirão Preto, 14096-350, Ribeirão Preto, SP, Brazil

[5]Fund for Citrus Protection, Fundecitrus, 14807-040, Araraquara, SP, Brazil

[6]School of Pharmacy & Biomolecular Sciences, Liverpool John Moores University, L3 3AF, Liverpool, UK

[a]These authors contributed equally to this work

*Corresponding author: University of São Paulo, School of Pharmaceutical Sciences of Ribeirão Preto, 14040-903, Ribeirão Preto, SP, Brazil. Tel: +55 16 33154723

e-mail address: gbraga@fcfrp.usp.br

ABSTRACT

The widespread use of conventional chemical antifungal agents has led to worldwide concern regarding the selection of resistant isolates. In this scenario, antimicrobial photodynamic treatment (APDT) has emerged as a promising alternative to overcome this issue. The technique is based on the use of a photosensitizer (PS) and light in the presence of molecular oxygen. Under these conditions, the PS generates reactive oxygen species which damage the biomolecules of the target organism leading to cell death. The great potential of APDT against plant-pathogenic fungi has already been reported both *in vitro* and *in planta*, indicating this control measure has the potential to be widely used in crop plants. However, there is a lack of studies on environmental risk with ecotoxicological assessment of PSs used in APDT. Therefore, this study aimed to evaluate the environmental toxicity of four phenothiazinium PSs: i) methylene blue (MB), ii) new methylene blue N (NMBN), iii) toluidine blue O (TBO), and iv) dimethylmethylene blue (DMMB) and also of the commercial antifungal NATIVO®, a mixture of trifloxystrobin and tebuconazole. The experiments were performed with *Daphnia similis* neonates and zebrafish embryos. Our results showed that the PSs tested had different levels of toxicity, with MB being the less toxic and DMMB being the most. Nonetheless, the environmental toxicity of these PSs were lower when compared to that of NATIVO®. Furthermore, estimates of bioconcentration and of biotransformation half-life indicated that the PSs are environmentally safer than NATIVO®. Taken together, our results show that the toxicity associated with phenothiazinium PSs would not constitute an impediment to their use in APDT. Therefore, APDT is a promising approach to control plant-pathogenic fungi with reduced risk for selecting resistant isolates and lower environmental impacts when compared to commonly used antifungal agents.

Keywords: ecotoxicity; fungicides; photodynamic treatment; photosensitizers; pollutants

1. INTRODUCTION

Pathogen resistance to antimicrobials is a major threat to global health (Perlin *et al.*, 2017). As a consequence, there is an ongoing and persistent search for new antimicrobials that could overcome such resistance. In this scenario, antimicrobial photodynamic treatment (APDT) has been presented as a promising alternative to control pathogens (Sabino *et al.*, 2020; Wainwright *et al.*, 2017). APDT is a therapy based on the use of three main components, namely a photosensitizer (PS), light, and molecular oxygen. The technique consists of applying a PS that preferentially binds to target cells followed by illumination with light of the appropriate wavelength. This will result in an excited PS molecule which will then react with molecular oxygen via either electron or energy transfer, generating reactive oxygen species (ROS) that will inactivate the target pathogen with little to no damage to the host (Castano *et al.*, 2004; Marasini *et al.*, 2021).

The efficiency of APDT has been shown for a variety of fungi and bacteria (Wainwright *et al.*, 2017). Reproductive fungal structures, such as conidia, are easily inactivated by APDT (de Menezes *et al.*, 2014a, 2014b, 2016; Gonzales *et al.*, 2017; Tonani *et al.*, 2018), which also overcomes multidrug-resistance in bacteria (Hamblin, 2016; Sabino *et al.*, 2020). Even *Deinococcus radiodurans*, a bacterium known for its remarkable tolerance to abiotic stressors and its potent antioxidant system, cannot withstand the damages caused by APDT (Nitzan and Ashkenazi, 1999). The emergence of resistance to APDT itself has been a topic of some studies (Kashef and Hamblin, 2017). The production of ROS that will nonspecifically react with and damage proteins, lipids, and nucleic acids leaves little room for known resistance mechanisms (Sabino *et al.*, 2020; Marasini *et al.*, 2021). However, it is important to mention that some recent studies have reported the emergence of tolerance to APDT in bacteria under specific conditions of sub-lethal treatment (Pieranski *et al.*, 2020; Rapacka-Zdonczyk *et al.*, 2019).

Several uses and applications of APDT have been proposed due to its efficiency against pathogens and its safety to the host, from treatment of mycoses to food decontamination (do Prado-Silva *et al.*, 2022; Wainwright *et al.*, 2017). One promising application of APDT is to control phytopathogenic fungi in crop fields (de Menezes *et al.*, 2014a, 2014b, 2016; Gonzales *et al.*, 2017). An important plant disease affecting *Citrus* species and resulting in extensive agricultural and economical losses is post-bloom fruit drop (PFD), which is caused by the fungus *Colletotrichum abscissum* (Dowling *et al.*, 2020; Gonçalves *et al.*, 2021; Peres *et al.*, 2005). PFD may decrease sweet orange production by as much as 80% (Silva-Junior *et al.*, 2014). Control of PFD is achieved via preventive spraying of antifungal agents during the blossoming period (Gama *et al.*, 2020; Silva-Junior *et al.*, 2014). However, only a small number of antifungals are approved for this use. For instance, in Brazil, only strobilurin and triazole antifungals are allowed on sweet orange commercial orchards (Silva-Junior *et al.*, 2014). This reduced variety of antifungal agents associated with their constant use presents the risk of selecting resistant strains, making PFD control less efficient (Dowling et al., 2020). Therefore, control of PFD in crop plants in an important example of a field that would benefit from APDT.

However, this use of APDT will invariably lead to contamination of soil and water with PSs. Therefore, the assessment of PS toxicity becomes a necessary step in order to safely use APDT in both crops and for food decontamination. Regulatory agencies require that compounds be tested with organisms from different trophic levels, such as producers and consumers, that also occupy distinct ecological niches (Bori *et al.*, 2016; Rila and Eisentraeger, 2003). In general, initial toxicology studies are performed in cultured cells. Although cell assays are useful in providing important background information regarding the molecules tested, they may not replace more in-depth experiments with

environmentally relevant organisms, such as microcrustaceans and fish (Bori *et al.*, 2016; Heger *et al.*, 2018; Rocha *et al.*, 2017).

Therefore, this work presents a toxicological assessment of four phenothiazinium PSs: i) methylene blue, ii) new methylene blue N, iii) toluidine blue O, and iv) dimethylmethylene blue and of the commercial product NATIVO®, a commonly used antifungal agent composed by a mixture of 10% trifloxystrobin and 20% tebuconazole. Our assessment comprised toxicity to the microcrustacean *Daphnia similis* and to embryos of zebrafish (*Danio rerio*) to better understand how the use of APDT may impact the environment when compared to conventional antifungal agents.

2. MATERIALS AND METHODS

2.1 Phenothiazinium photosensitizers

The four phenothiazinium PSs used in the present work were: methylene blue (MB, Cat# M9140), new methylene blue N (NMBN, Cat# 202096), toluidine blue O (TBO, Cat# T3260), and dimethylmethylene blue (DMMB, Cat# 341088) (Supplementary Figure 1A), all purchased from Sigma. Concentrations used varied for each experiment type and are specified below.

2.2 NATIVO®

The fungicides belonging the groups of quinone outside inhibitors (QoI) and demethylation inhibitors (DMI) have been the most used for disease control in different crops (Oliver & Hewitt, 2014). The commercial antifungal agent NATIVO® (Bayer CropScience) is a 2:1 mixture of a DMI, trifloxystrobin (100 g L^{-1}), and of a QoI, tebuconazole (200 g L^{-1}) (Supplementary Figure 1B). The original product was diluted to obtain final concentrations of trifloxystrobin and tebuconazole of 40 and 80 mg L^{-1},

130 respectively. This dilution corresponds to the concentration applied in the field for the
131 control of phytopathogenic fungi. Then, a series of 1:10 dilutions (10^{-1} to 10^{-8}) were
132 performed, always in distilled water. Dilutions used in each experiment varied and are
133 specified below.
134
135 **2.3 Ecotoxicity assessments with *Daphnia similis***
136 The assays with *D. similis* were performed according to the ABNT NBR 12713
137 guidelines for aquatic ecotoxicology assessment ("Ecotoxicologia aquática – Toxicidade
138 aguda – Método de ensaio com *Daphnia* spp", 2016). *D. similis* was kept in 1-L containers
139 at 20 ± 2 °C with a maximum of 25 organisms per container. Diffuse illumination was
140 provided in 12:12h photoperiod with an irradiance of 1000 lux. The organisms were fed
141 with the alga *Pseudokirchneriella subcaptata* (3×10^6 cells/organism). Culture medium
142 was replaced every two weeks and the organisms were maintained for up to 28 days.
143 Ecotoxicological assessment was performed with *D. similis* neonates aged
144 between 6 and 24 h and obtained via parthenogenesis. Each treatment consisted of four
145 replicate groups with five organisms each. Exposure to the PS was performed at 20 ± 2
146 °C for 48 h. No feeding was allowed during the experiment. Concentrations of PS used
147 in these experiments were 0.3125, 0.625, 1.25, 2.5, and 5 µM, which were chosen based
148 on a preliminary experiment to assess the concentration interval and specific points. The
149 effect of light on toxicity was assessed by performing the 48-h incubation under a 12:12
150 h light:dark photoperiod. Then, the numbers of mobile and immobile individuals were
151 counted. The half-maximum effective concentration (EC_{50}) was calculated by the
152 trimmed Spearman-Karber method based on data from three independent experiments.
153
154 **2.4 Ecotoxicity assessment with *Danio rerio* embryos**

155 The experiments with zebrafish were approved by the institution's Animal Ethics
156 Committee (Protocol No. 18.1.496.60.1). Adult organisms were maintained and used
157 following the guidelines of the test No. 236 of the Organisation for Economic Co-
158 operation and Development (OECD) Guidelines for the Testing of Chemicals (OECD,
159 2013) in a ZEBTEC system (Tecniplast, Italy) at 26 ± 1 °C with a 14:10h (light:dark)
160 photoperiod. Fish were fed twice a day with Tetramin® (Tetra GmbH, Germany). Eggs
161 were obtained by placing adult fish at a 2:1 male:female ratio to allow for breeding. Thirty
162 minutes after laying, eggs were collected, transferred to a petri dish and washed with
163 distilled water. Only eggs that had achieved the stage of blastula were used for the
164 experiments.

165 Fertilized eggs were exposed to PS in increasing concentrations (1, 10, 25, 50, and
166 100 µM) and to five successive 10-fold dilutions of the commercial antifungal NATIVO®
167 starting at 40 mg L^{-1} trifloxystrobin and 80 mg L^{-1} tebuconazole. Exposure was performed
168 in 24-well plates at 26 ± 1 °C for 144 h. A total of 20 embryos was used for each condition.
169 Development was assessed 24, 48, 72, 96, 120, and 144 h after exposure had commenced.
170 A stereo microscope (SMZ-800, Nikon) coupled to a digital camera was used to evaluate
171 parameters pertaining to lethality (egg coagulation, malformation, non-detachment of the
172 embryo tail, and absence of heart beat), to sub-lethality (eye development, spontaneous
173 coiling, pigmentation, and edema formation), and to teratogenicity (heart and tail
174 malformations, non-inflation of the swim bladder, pericardial edema, yolk sac edema, and
175 skeletal deformities). To assess the effects of light on toxicity, 24-well plates were placed
176 under a 14:10 h light:dark photoperiod for the duration of the experiments. For dark
177 toxicity, plates were covered in aluminum foil and placed inside the same chamber.
178 Positive controls were run in parallel to each experiment by treatment samples with 4 mg
179 L^{-1} 3,4-dichloroaniline (Sigma). Half maximum lethal concentrations (LC_{50}) were

180 calculated with a four-parameter logistic regression using Prism 8 software (GraphPad
181 Software).
182
183 **2.5 Bioconcentration factor and biotransformation half-life**
184 Bioconcentration factor (BCF) and biotransformation half-life in fish were
185 calculated with EPIWEB 4.1 software (EPA – Environmental Protection Agency). BCF
186 was estimated using the equation:
187 $$log\ BCF = 0.6598\ logP - 0.333 \quad (1)$$
188 where *P* is the octanol/water partition coefficient as calculated by MarvinJS logD
189 Predictor software (ChemAxon).
190
191 **2.6 Statistical analyses**
192 All statistical analyses were performed with Prism 8 software (GraphPad
193 Software). Student's *t*-test were used for pairwise comparisons at a significance level of
194 0.05. Analysis of variance (ANOVA) was used for multiple comparisons with Tukey's
195 post-test also set to a significance level of 0.05.
196
197 **3. RESULTS AND DISCUSSION**
198 Many studies have previously reported the high efficiency of APDT as a technique
199 to control plant pathogenic fungi both *in vitro* and *in planta* (de Menezes *et al.*, 2014a,
200 2014b; Fracarolli *et al.*, 2016; Gonzales *et al.*, 2017). For instance, APDT with
201 phenothiazines (in the range of 10-50 µM) against *C. abscissum* can achieve nearly
202 complete inactivation in under one hour of red light exposure (de Menezes *et al.*, 2014b).
203 Furthermore, efficient *in planta* inactivation of *C. abscissum* is possible with MB at 50
204 µM after only 30 min of solar exposure (Gonzales *et al.*, 2017). Importantly, this *in planta*

inactivation does not result in damage to the host plant (Gonzales *et al.*, 2017). Additionally, and unlike traditional antifungals, APDT can inactivate dormant structures such as conidia. However, an ecotoxicological assessment of PSs and a comparison with commonly used antifungal agents is still lacking.

Initially, we performed ecotoxicological experiments with the microcrustacean *D. similis*, representing a low trophic level organism. Toxicity to *D. similis* was calculated based on the number of mobile and immobile individuals after exposure to all PSs (in the dark and under light) and to the antifungal agent NATIVO®. The PS DMMB was the most toxic among the PSs tested with an EC_{50} of 1.0 µM in the dark (Table 1). The other three PSs (MB, NMBN, and TBO) were less toxic than DMMB but presented similar toxicity between them (2.2, 2.01, and 2.6 µM, respectively) (Table 1). For all PSs tested, we observed no difference between experiments performed in the dark and under light (Table 1). This result may be a consequence of the high toxicity levels already observed in the dark. In this situation, light exposure and subsequent ROS production may not significantly increase mortality. More importantly, the antifungal agent NATIVO® caused mortality of all *D. similis* neonates at every dilution tested, thus preventing the calculation of an EC_{50} value and indicating that any of the PSs tested present a lower environmental risk when compared to the commercial antifungal.

Table 1 – Average half-maximum effective concentration (EC_{50}) for the indicated photosensitizers obtained in *Daphnia similis* neonates. Values were obtained in the dark or under light exposure. The antifungal NATIVO® caused total mortality of all neonates, thus preventing the calculation of an EC_{50}

*different upper-case letters indicate significant difference between dark or light treatments for the same photosensitizer; whereas different lower-case letters indicate significant difference between different photosensitizers under the same exposure conditions (Tukey's test, $P < 0.05$)

EC_{50}	(µM)		(mg L^{-1})		GHS Category
Photosensitizer	**Dark**	**Light**	**Dark**	**Light**	**(Acute Aquatic Toxicity)**
Methylene Blue	$2.2 \pm 0.2^{A,a}$*	$2.1 \pm 0.6^{A,a}$	$0.82 \pm 0.07^{A,a}$*	$0.8 \pm 0.2^{A,a}$	1
New Methylene Blue	$2.01 \pm 0.04^{A,a}$	$2.0 \pm 0.4^{A,a}$	$0.84 \pm 0.02^{A,a}$	$0.8 \pm 0.2^{A,a}$	1
Toluidine Blue O	$2.6 \pm 0.5^{A,a}$	$2.9 \pm 0.1^{A,a}$	$0.8 \pm 0.2^{A,a}$	$0.89 \pm 0.03^{A,a}$	1
Dimethylmethylene Blue	$1.0 \pm 0.4^{A,b}$	$0.8 \pm 0.3^{A,b}$	$0.4 \pm 0.2^{A,b}$	$0.3 \pm 0.1^{A,b}$	1

231 Furthermore, based on the calculated EC_{50} values, all the PSs are classified as
232 category 1 (very toxic to aquatic life, i.e. $EC_{50} \leq 1$ mg/l) following GHS criteria (Table
233 1). Even though no EC_{50} value could be obtained for NATIVO®, the observed mortality
234 of all neonates is a good indication of higher toxicity.

235 We then performed an ecotoxicological assessment in embryos of *D. rerio*, an
236 organism representing a high trophic level. Acute toxicity to zebrafish embryos was
237 assessed according to Test No. 236 from the OECD for all PSs (both in the dark and under
238 light) and for the antifungal agent NATIVO®.

239 The PS MB presented no mortality to embryos, indicating low acute toxicity (Fig.
240 1A). Furthermore, emerging larvae only presented significant issues with swim bladder
241 inflation at 100 µM (Fig. 1C and Fig. 2A and 2B). There were no significant statistical
242 differences between dark (Fig. 1A and 1C) and light (Fig. 1B and 1D) treatments for both
243 mortality and swim bladder inflation issues. However, exposure to MB resulted in larval
244 scoliosis as well as pericardial and yolk sac edema, but these were only observed at the
245 highest concentration of 100 µM and occurred exclusively under illumination (Fig. 2C
246 and 2D).

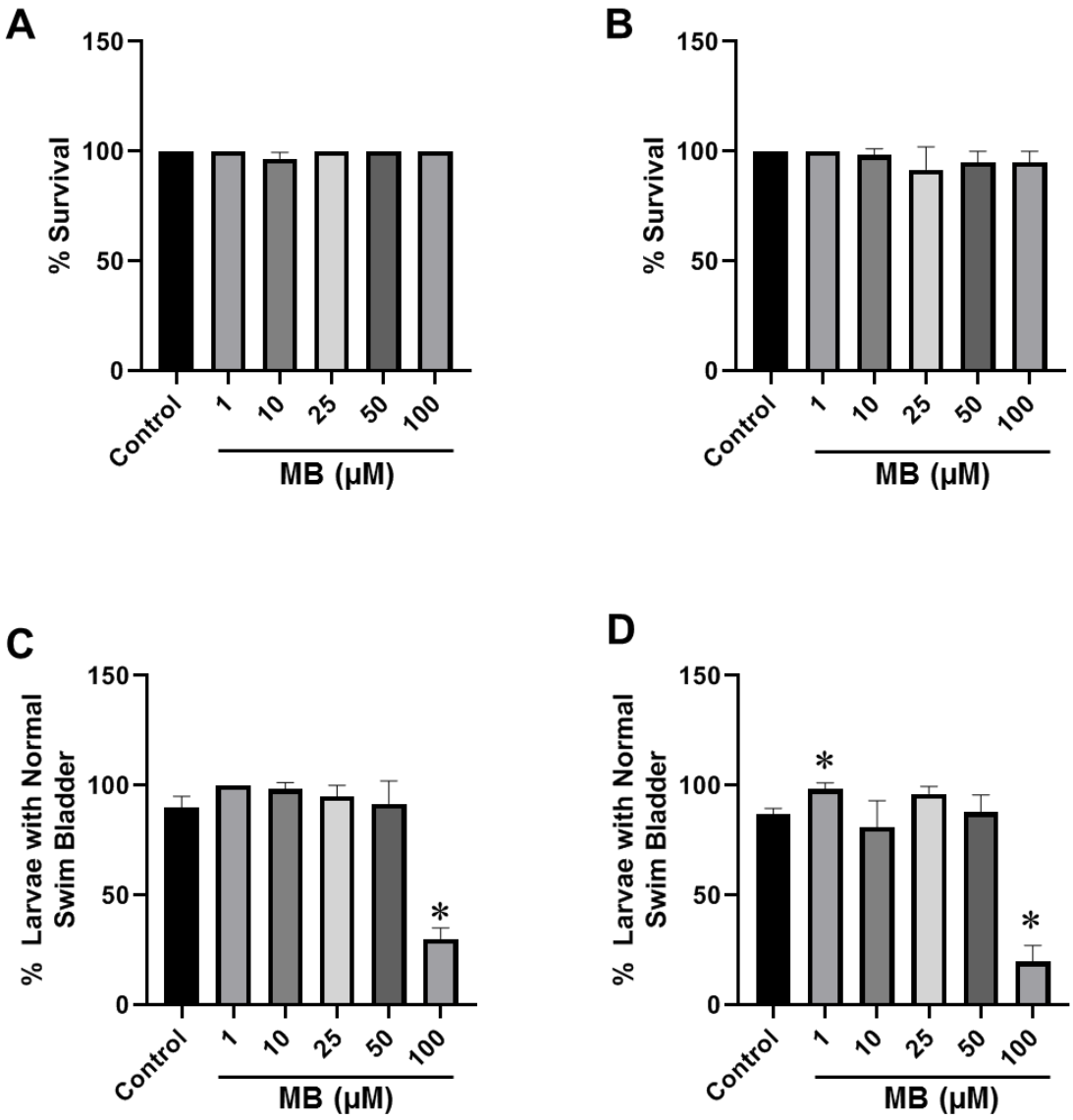


Figure 1 – Toxicity of the photosensitizer methylene blue (MB) on embryos of *Danio rerio*. Acute toxicity was evaluated by measuring (**A** and **B**) mortality and (**C** and **D**) the ability of surviving larvae to inflate the swim bladder. Assessment was performed both in the dark (**A** and **C**) and under light (**B** and **D**). Values are mean and error bars are standard deviation from three independent experiments. Asterisks indicate that means are statistically different from the control group

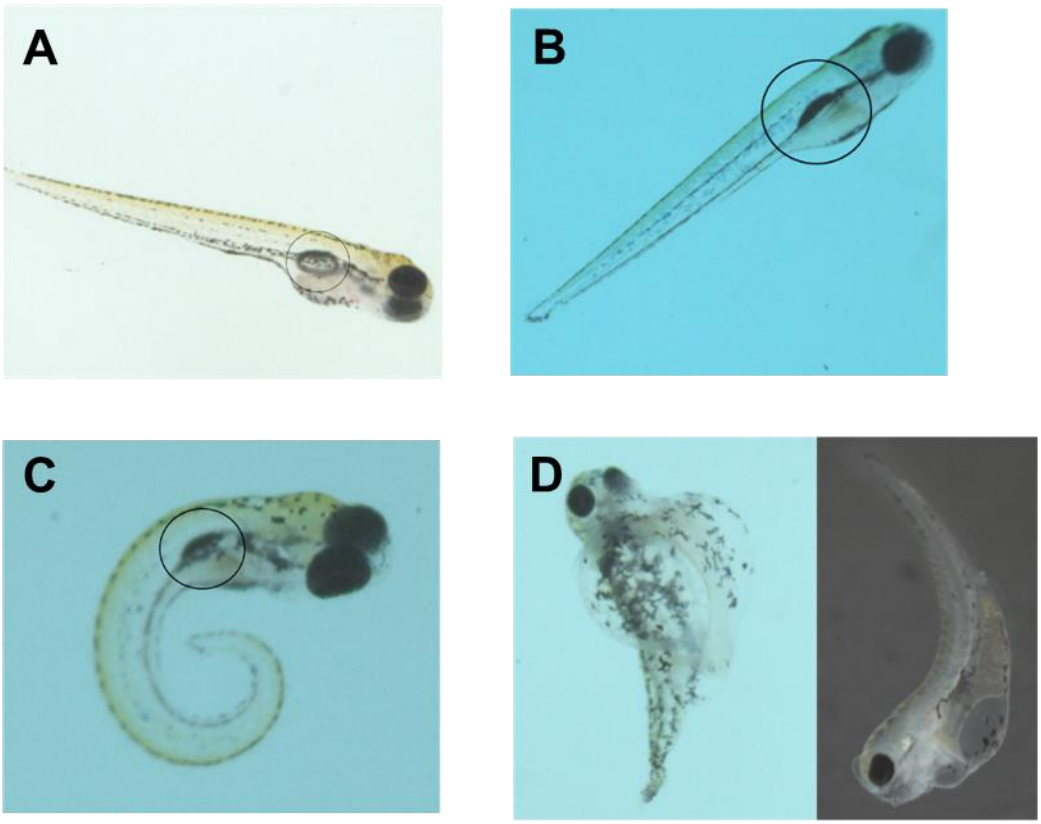
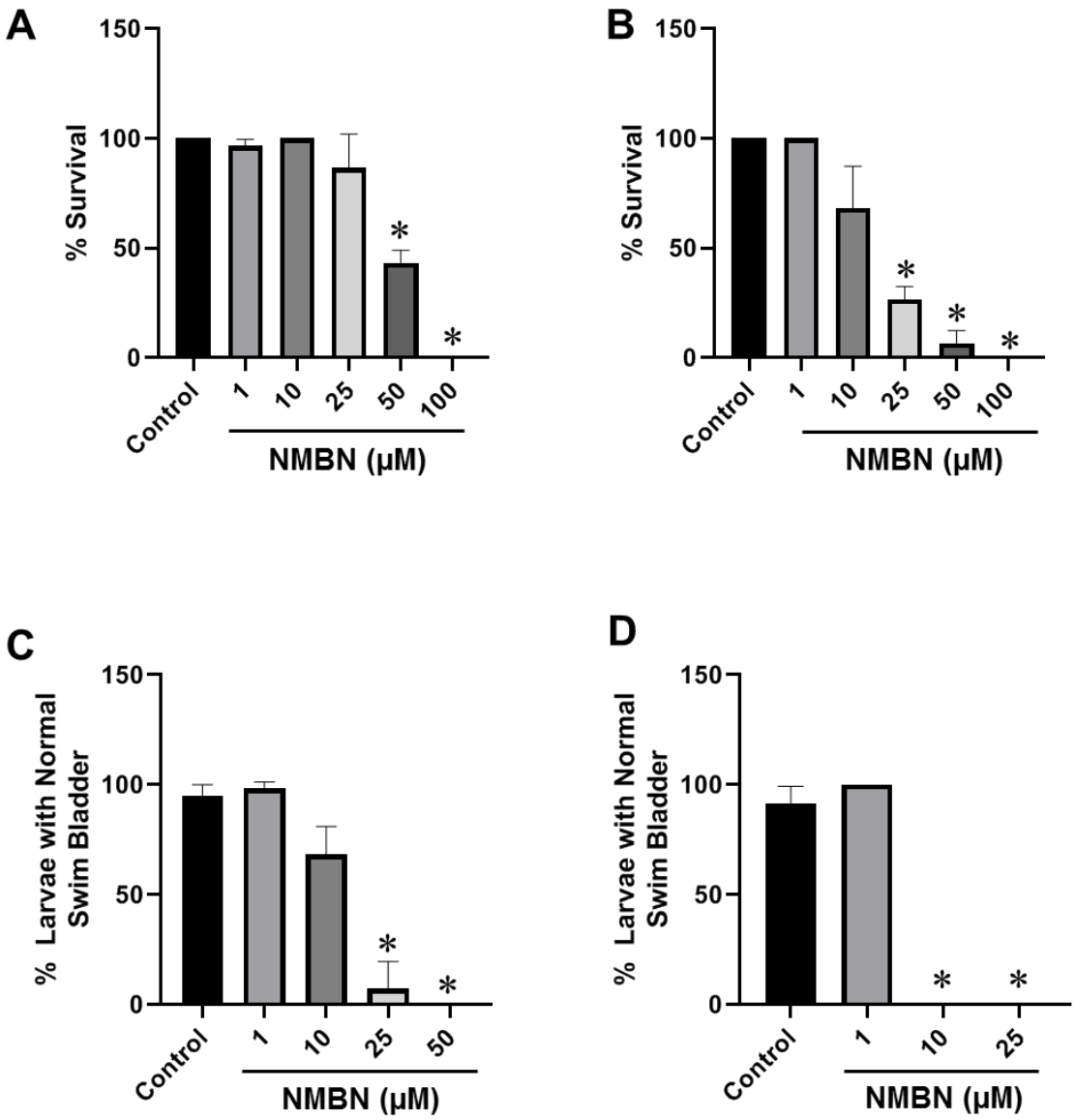


Figure 2 – The effects of the photosensitizer methylene blue (MB) on *Danio rerio* larvae. (**A**) Larva from the negative control showing normal development and an inflated swim bladder. (**B**) A non-inflated swim bladder caused by MB at 100 µM. (**C**) Scoliosis caused by MB at 100 µM in the presence of light. (**D**) Pericardial and yolk sac edema caused by MB at 100 µM under illumination

For NMBN, unlike reported for MB, it was possible to observe an effect of light exposure. Significant mortality was observed at 50 µM in the dark, but a similar result was already observed at 25 µM under illumination (Fig. 3A and 3B). Similarly, non-inflated swim bladders were observed at 25 µM in the dark, but at only 10 µM in the presence of light (Fig. 3C and 3D). Calculated LC_{50} values for NMBN were 49.8 µM in the dark and 15.4 µM under illumination (Table 2).



267

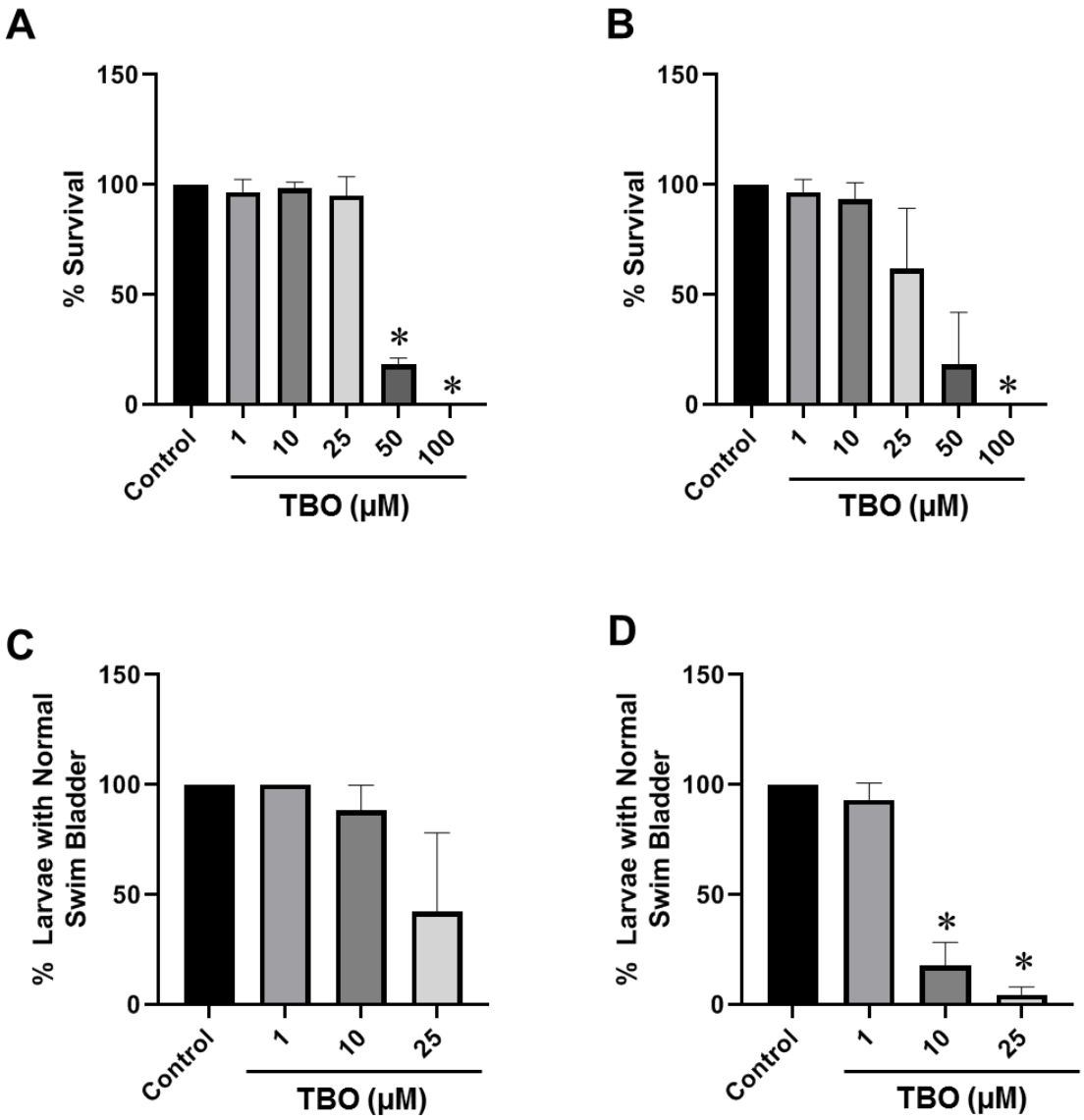
268 **Figure 3 –** Toxicity of the photosensitizer new methylene blue N (NMBN) on embryos of *Danio*
269 *rerio*. Acute toxicity was evaluated by measuring (**A** and **B**) mortality and (**C** and **D**) the ability
270 of surviving larvae to inflate the swim bladder. Assessment was performed both in the dark (**A**
271 and **C**) and under light (**B** and **D**). Values are mean and error bars are standard deviation from
272 three independent experiments. Asterisks indicate that means are statistically different from the
273 control group

274

Table 2 – Average half-maximum lethal concentration (LC_{50}) for the indicated photosensitizers obtained in *Danio rerio* embryos. Values were obtained in the dark or under light exposure. For reference, NATIVO® is registered as GHS category 1

LC_{50}	(μM)		(mg L^{-1})		GHS Category (Acute Aquatic Toxicity)	
Photosensitizer	**Dark**	**Light**	**Dark**	**Light**	**Dark**	**Light**
Methylene Blue	> 100	> 100	> 37.4	> 37.4	-	-
New Methylene Blue	49.8	15.4	20.7	6.4	3	2
Toluidine Blue O	40.5	31.2	12.4	9.5	3	2
Dimethylmethylene Blue†	1-10	1-10	0.416-4.16	0.416-4.16	1-2	1-2

For the PS TBO, light exposure did not significantly affect mortality to embryos (Fig. 4A and 4B), although there was a tendency toward some light effect with LC_{50} values being 40.5 μM in the dark and 31.2 μM after light exposure (Table 2). Indeed, light was observed to influence swim bladder inflation because non-inflated swim bladders occurred exclusively under illumination (Fig. 4C and 4D).

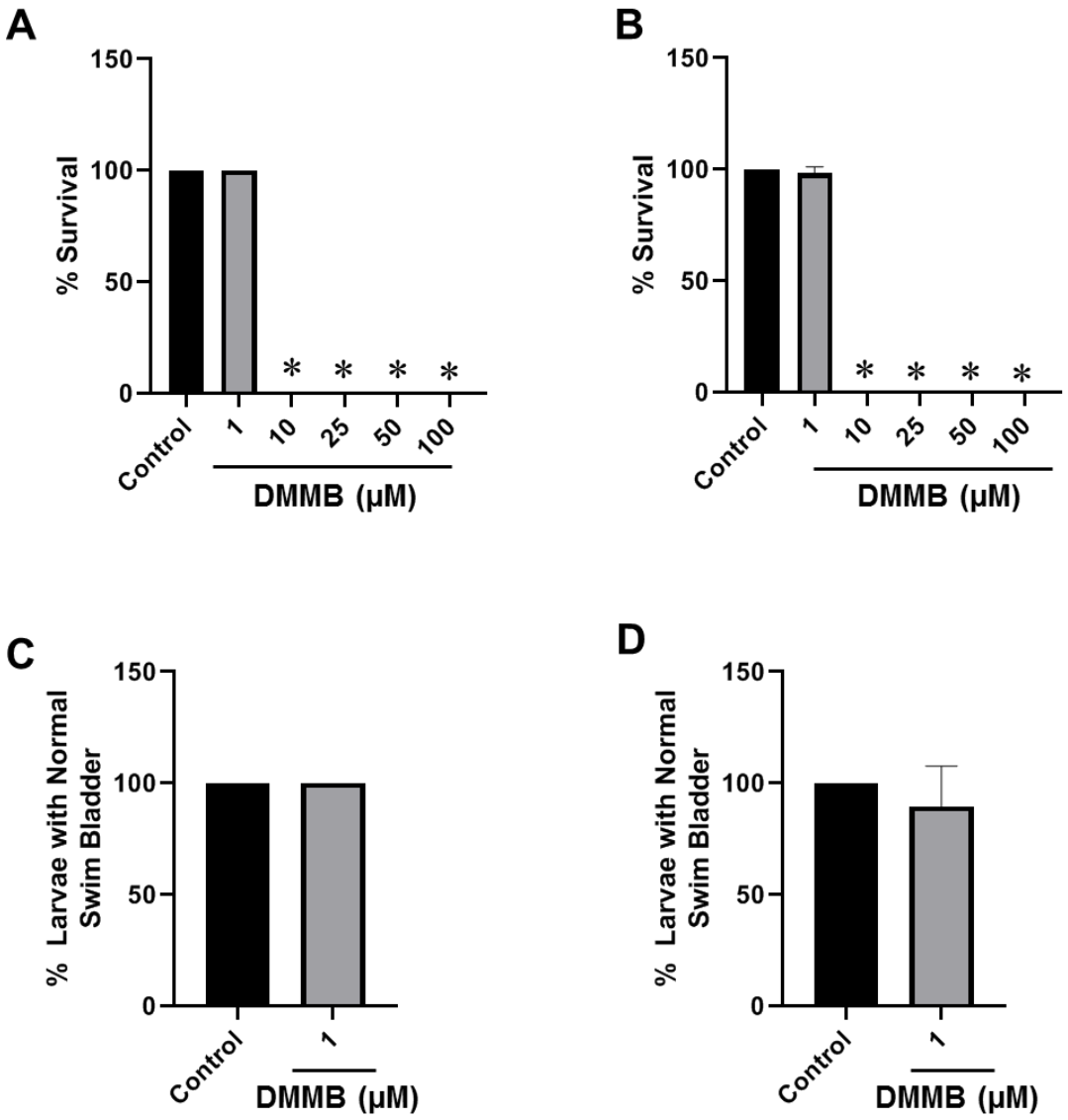


284

285 **Figure 4 –** Toxicity of the photosensitizer toluidine blue O (TBO) on embryos of *Danio rerio*.
286 Acute toxicity was evaluated by measuring (**A** and **B**) mortality and (**C** and **D**) the ability of
287 surviving larvae to inflate the swim bladder. Assessment was performed both in the dark (**A** and
288 **C**) and under light (**B** and **D**). Values are mean and error bars are standard deviation from three
289 independent experiments. Asterisks indicate that means are statistically different from the control
290 group

291

292 The PS DMMB once again presented the highest toxicity among the PSs tested.
293 Concentrations as low as 10 µM were sufficient to cause 100% mortality of embryos (Fig.
294 5A and 5B). The only relatively safe concentration of DMMB was 1 µM, for which no
295 mortality (Fig. 5A and 5B) and no negative effects on the swim bladder (Fig. 5C and 5D)
296 were observed.



297

298 **Figure 5 –** Toxicity of the photosensitizer dimethylmethylene blue (DMMB) on embryos of
299 *Danio rerio*. Acute toxicity was evaluated by measuring (**A** and **B**) mortality and (**C** and **D**) the
300 ability of surviving larvae to inflate the swim bladder. Assessment was performed both in the dark
301 (**A** and **C**) and under light (**B** and **D**). Values are mean and error bars are standard deviation from
302 three independent experiments. Asterisks indicate that means are statistically different from the
303 control group

304

305 The commercial antifungal agent NATIVO® caused 100% mortality even when
306 used at a 10^{-3} dilution (Fig. 6A), which corresponds to trifloxystrobin and tebuconazole
307 concentrations of 0.04 and 0.08 mg L^{-1}, respectively. Dilutions of 10^{-4} and 10^{-5} allowed
308 embryos to survive and caused no negative effects on swim bladders (Fig. 6A and 6B).

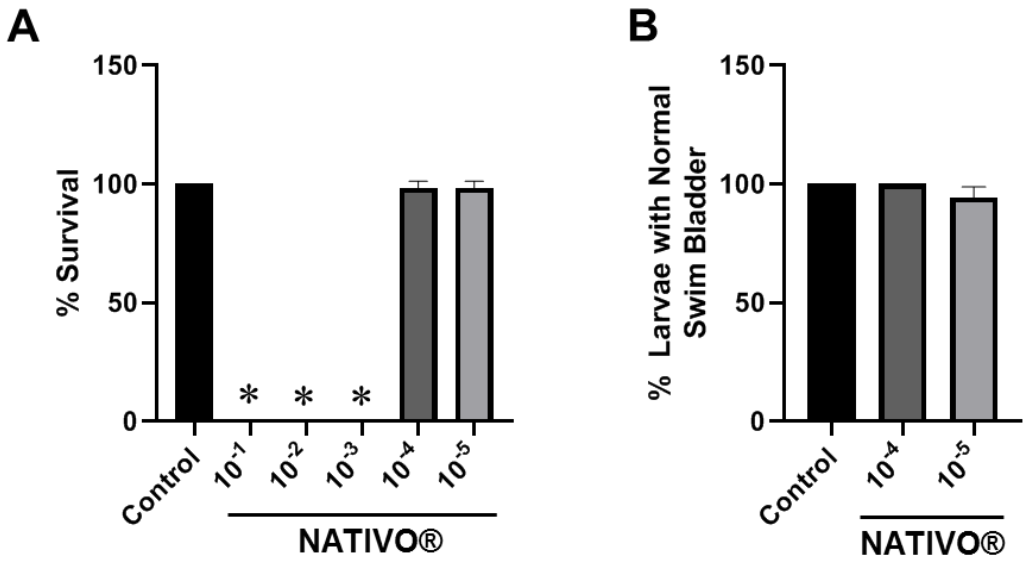


Figure 6 – Toxicity of the commercial antifungal agent NATIVO® on embryos of *Danio rerio*. Acute toxicity was evaluated by measuring (**A**) mortality and (**B**) the ability of surviving larvae to inflate the swim bladder. Values are mean and error bars are standard deviation from three independent experiments. Asterisks indicate that means are statistically different from the control group

Based on calculated LC_{50} values for all PSs (Table 2), both NMBN and TBO are classified as GHS category 3 in the dark and category 2 under light, showing that illumination is an important determinant of environmental toxicity for these PSs. For MB, no classification was possible because mortality levels never reached 50%. The highest concentration tested for MB was 100 µM, which represents 37.4 mg L^{-1}. Therefore, there is still room for MB to be classified as GHS category 3 if mortality rates of 50% are achieved before the 100 mg L^{-1} threshold. Finally, for DMMB, no precise calculation of LC_{50} was possible because mortality increased from 0 to 100% for two adjacent concentrations (1 and 10 µM). However, this places the LC_{50} value between 0.416 and 4.16 mg L^{-1}, resulting in classification as either category 1 or 2 (Table 2). The antifungal NATIVO®, as a commercial product, is already classified as GHS category 1 by the manufacturer.

Considering the results from the two assays, namely those with *D. similis* neonates and with *D. rerio* embryos, we can tentatively classify all tested compounds in the following order of environmental risk, from lowest to highest: MB < TBO < NMBN < DMMB < NATIVO®.

Finally, to compare the potential of both PSs and NATIVO® to bioconcentrate in fish, we mathematically estimated BCF and biotransformation half-life. Less lipophilic PSs such as MB, NMBN, and TBO had BCF values ranging from 12.9 to 50.0 L kg^{-1} (Table 3). The more lipophilic PS DMMB and the fungicide tebuconazole displayed BCF values of 117 and 126 L kg^{-1}, respectively. Accordingly, trifloxystrobin, as the most lipophilic molecule, had a BCF value of 682 L kg^{-1} (Table 3), indicating a higher potential to bioconcentrate when compared to all the PSs and to tebuconazole.

Table 3 – Estimates of bioconcentration factor (BCF) and biotransformation half-life as obtained from the Environmental Protection Agency EPIWEB 4.1 software

[a]*P* is the octanol/water partition coefficient as calculated by MarvinJS logD Predictor

[b]BCF was calculated using Eq. (1) (see Materials and Methods)

[c]normalized to 10 g of fish at 15 °C

Molecule	log *P* (pH 7.0)[a]	BCF (L kg^{-1})[b]	Biotransformation half-life (days)[c]
Methylene Blue	2.61	24.5	0.11
New Methylene Blue	3.08	50.0	1.1
Toluidine Blue O	2.19	12.9	3.6×10^{-3}
Dimethylmethylene Blue	3.64	117.0	1.3
Trifloxystrobin	4.80	682.0	2.8
Tebuconazole	3.69	126.0	5.1

We also estimated biotransformation half-life in fish with EPIWEB 4.1 software. Tebuconazole and trifloxystrobin presented half-lives of 5.1 and 2.8 days, respectively (Table 3). Both of these values exceed the estimated half-life of DMMB, which had the longest half-life (1.3 days) among all PSs (Table 3). The PSs MB and TBO, being the less

lipophilic and simplest molecules, had half-life values of 0.11 and 0.0036 days, respectively (Table 3). Although these data are the result of estimates, there is enough information in the literature to support the idea that both trifloxystrobin and tebuconazole accumulate in organisms and in the environment. Trifloxystrobin was found to bioaccumumlate in *Gobiocypris rarus* embryos (Zhu *et al.*, 2015). Furthermore, trifloxystrobin can be metabolized in soil to yield trifloxystrobin acid, a molecule with increased half-life and that was shown to greatly accumulate in the earthworm *Eisenia fetida* (Liu *et al.*, 2020). Regarding tebuconazole, it was reported to bioaccumulate in *Cyprinus carpio* muscle (Clasen *et al.*, 2018). Also, removal of tebuconazole from water may be problematic as a conventional drinking-water treatment plant was reported to be unable to completely remove tebuconazole from river water samples (Elfikrie *et al.*, 2020). In accordance, tebuconazole is the most prevalent fungicide in surface water (de Souza *et al.*, 2020).

One aspect that needs to be considered is the stability of PSs in the environment. In this regard, a previous study from our research group has reported that phenothiazinium PSs exposed to sunlight steeply lose their effectiveness (de Menezes *et al.*, 2014b). For instance, new methylene blue N loses 99.9% of its inactivation efficiency against *C. abscissum* after 12 h of sunlight exposure. This reduction is accompanied by a flattening of the absorption spectrum in the visible range (i.e., photobleaching) (de Menezes *et al.*, 2014b). In our study, we used 'naïve' (i.e. not previously exposed to light) photosensitizers because using photobleached ones would likely lead to reduced toxicity under illumination. Additionally, we can speculate that photosensitizers reaching the environment from crop plants would have already been exposed to considerable amounts of solar radiation. If this assumption is correct, ecotoxicity in real world applications would not be as high as the values obtained under light exposure conditions in this study.

When compared to trifloxystrobin and tebuconazole, the PS MB has lower toxicity, lower BCF and a much shorter biotransformation half-life (Table 3). Also, our research group has previously reported that MB can be used at 50 µM to efficiently inactivate *C. abscissum* in plants (Gonzales *et al.*, 2017). This concentration is below the LC_{50} values obtained for zebrafish embryos both in the dark and under illumination (Table 2). However, a concentration of 50 µM is well above the EC_{50} values for *D. similis* immobilization (Table 1). Nonetheless, it is important to note that using 50 µM (18.7 mg L^{-1} in the case of MB) to treat crop plants would likely not result in such a high final concentration in water bodies. For instance, the highest concentration of antibiotics in effluent water samples obtained from pharmaceutical manufacturers was found to be 252 µg L^{-1}, and this concentration is higher compared to those obtained for hospital and aquaculture effluents (Thai *et al.*, 2018). Such reduced toxicity, combined with the fact that an MB injection is approved by both the Food and Drug Administration (NDA204630) and the European Medicines Agency (EMA/H/C/002108) for the treatment of methemoglobinemia, makes MB the most likely candidate to obtain approval for other applications. Of course, the use of MB is not without its own accumulation issues (Krishna Moorthy *et al.*, 2021; Park, Baek and Moon, 2019; Rifici *et al.*, 1996), but diverse and effective methods of removing MB from water are abundant and up-to-date (Gouamid *et al.*, 2013; Hoslett *et al.*, 2020; Mantasha *et al.*, 2020; Reema *et al.*, 2011; Somsesta *et al.*, 2020).

Even though MB was the least toxic PS as long as environmental risk is concerned, the other PSs should not be immediately deemed unsuitable for use. This is because circumstances may dictate which PS ought to be used. For instance, NMBN is a more potent PS when compared to MB (Rodrigues *et al.*, 2013; Wainwright *et al.*, 1998), which

would likely translate into smaller dose requirements, leading to lower levels of environmental contamination.

CONCLUSION

Our results provide a comprehensive view of the environmental risk associated with the use of diverse PS. The environmental consequences associated with PS use are diminished when compared to currently approved and widely used antifungal agents, such as NATIVO®. Therefore, environmental risk should not be a barrier in the path of using APDT to control plant-pathogenic fungi in the future.

CONFLICT OF INTEREST

This article does not necessarily reflect the views of CETESB and no official endorsement should be inferred.

ACKNOWLEDGEMENTS

We thank Leandro F. Pippa (Faculdade de Ciências Farmacêuticas de Ribeirão Preto, USP) for carefully reading the manuscript.

FUNDING

This work was supported by the State of São Paulo Research Foundation (FAPESP, Brazil; grants 2016/24269-7 to DCT; 2016/11386-5 to GULB; 2018/24298-2 and 2014/50945-4 to DPO; 2018/06945-0 to FRA), the National Council for Scientific and Technological Development (CNPq, Brazil; grants 165191/2020-1 to GTPB; 425998/2018-5 and 307738/2018-3 to GULB; 465571/2014-0 to DPO), and the

Coordination for the Improvement of Higher Education Personnel (CAPES; Finance Code 001).

REFERENCES

Bori, J., Vallès, B., Ortega, L. and Riva, M. C. (2016) Bioassays with terrestrial and aquatic species as monitoring tools of hydrocarbon degradation. *Environmental Science and Pollution Research*, 23, pp. 18694-18703.

Castano, A. P., Demidova, T. N. and Hamblin, M. R. (2004) Mechanisms in photodynamic therapy: part one—photosensitizers, photochemistry and cellular localization. *Photodiagnosis and Photodynamic Therapy*, 1, pp. 279-293.

Clasen, B., Loro, V. L., Murussi, C. R., Tiecher, T. L., Moraes, B. and Zanella, R. (2018) Bioaccumulation and oxidative stress caused by pesticides in *Cyprinus carpio* reared in a rice-fish system. *Science of The Total Environment*, 626, pp. 737-743.

de Menezes, H. D., Pereira, A. C., Brancini, G. T. P., de Leao, H. C., Massola Junior, N. S., Bachmann, L., Wainwright, M., Bastos, J. K. and Braga, G. U. L. (2014a) Furocoumarins and coumarins photoinactivate *Colletotrichum acutatum* and *Aspergillus nidulans* fungi under solar radiation. *Journal of Photochemistry and Photobiology B: Biology*, 131, pp. 74-83.

de Menezes, H. D., Rodrigues, G. B., Teixeira, S. P., Massola Jr., N. S., Bachmann, L., Wainwright, M. and Braga, G. U. L. (2014b) *In vitro* photodynamic inactivation of plant-pathogenic fungi *Colletotrichum acutatum* and *Colletotrichum gloeosporioides* with novel phenothiazinium photosensitizers. *Applied and Environmental Microbiology*, 80, pp. 1623-1632.

de Menezes, H. D., Tonani, L., Bachmann, L., Wainwright, M., Braga, G. Ú. L. and von Zeska Kress, M. R. (2016) Photodynamic treatment with phenothiazinium photosensitizers kills both ungerminated and germinated microconidia of the pathogenic fungi *Fusarium oxysporum*, *Fusarium moniliforme* and *Fusarium solani*. *Journal of Photochemistry and Photobiology B: Biology*, 164, pp. 1-12.

de Souza, R. M., Seibert, D., Quesada, H. B., de Jesus Bassetti, F., Fagundes-Klen, M. R. and Bergamasco, R. (2020) Occurrence, impacts and general aspects of pesticides in surface water: A review. *Process Safety and Environmental Protection*, 135, pp. 22-37.

454 do Prado-Silva, L., Brancini, G. T. P., Braga, G. Ú. L., Liao, X., Ding, T. and Sant'Ana,
455 A. S. (2022) Antimicrobial photodynamic treatment (aPDT) as an innovative technology
456 to control spoilage and pathogenic microorganisms in agri-food products: An updated
457 review. *Food Control*, 132, 108527.

458 Dowling, M., Peres, N., Villani, S. and Schnabel, G. (2020) Managing *Colletotrichum* on
459 fruit crops: a "complex" challenge. *Plant Disease,* 104, pp. 2301-2316.

460 Elfikrie, N., Ho, Y. B., Zaidon, S. Z., Juahir, H. and Tan, E. S. S. (2020) Occurrence of
461 pesticides in surface water, pesticides removal efficiency in drinking water treatment
462 plant and potential health risk to consumers in Tengi River Basin, Malaysia. *Science of*
463 *The Total Environment,* 712, 136540.

464 Fracarolli, L., Rodrigues, G. B., Pereira, A. C., Massola Junior, N. S., Silva-Junior, G. J.,
465 Bachmann, L., Wainwright, M., Bastos, J. K. and Braga, G. U. L. (2016) Inactivation of
466 plant-pathogenic fungus *Colletotrichum acutatum* with natural plant-produced
467 photosensitizers under solar radiation. *Journal of Photochemistry and Photobiology B:*
468 *Biology*, 162, pp. 402-411.

469 Gama, A. B., Baggio, J. S., Rebello, C. S., Lourenço, S. d. A., Gasparoto, M. C. d. G., da
470 Silva Junior, G. J., Peres, N. A. and Amorim, L. (2020) Sensitivity of *Colletotrichum*
471 *acutatum* isolates from citrus to carbendazim, difenoconazole, tebuconazole, and
472 trifloxystrobin. *Plant Disease*, 104, pp. 1621-1628.

473 Gonçalves, F. P., Nogueira Júnior, A. F., Silva-Junior, G. J., Ciampi-Guillardi, M. and
474 Amorim, L. (2021) Environmental requirements for infection of *Colletotrichum acutatum*
475 and *C. gloeosporioides* sensu lato in citrus flowers and prevalence of these pathogens in
476 Brazil. *European Journal of Plant Pathology*, 160, pp. 27-37.

477 Gonzales, J. C., Brancini, G. T. P., Rodrigues, G. B., Silva-Junior, G. J., Bachmann, L.,
478 Wainwright, M. and Braga, G. U. L. (2017) Photodynamic inactivation of conidia of the
479 fungus *Colletotrichum abscissum* on *Citrus sinensis* plants with methylene blue under
480 solar radiation. *Journal of Photochemistry and Photobiology B: Biology,* 176, pp. 54-61.

481 Gouamid, M., Ouahrani, M. R. and Bensaci, M. B. (2013) Adsorption equilibrium,
482 kinetics and thermodynamics of methylene blue from aqueous solutions using date palm
483 leaves. *Energy Procedia,* 36, pp. 898-907.

Hamblin, M. R. (2016) Antimicrobial photodynamic inactivation: a bright new technique to kill resistant microbes. *Current Opinion in Microbiology,* 33, pp. 67-73.

Heger, S., Du, M., Bauer, K., Schäffer, A. and Hollert, H. (2018) Comparative ecotoxicity of potential biofuels to water flea (*Daphnia magna*), zebrafish (*Danio rerio*) and Chinese hamster (*Cricetulus griseus*) V79 cells. *Science of The Total Environment,* 631-632, pp. 216-222.

Hoslett, J., Ghazal, H., Mohamad, N. and Jouhara, H. (2020) Removal of methylene blue from aqueous solutions by biochar prepared from the pyrolysis of mixed municipal discarded material. *Science of The Total Environment,* 714, 136832.

Kashef, N. and Hamblin, M. R. (2017) Can microbial cells develop resistance to oxidative stress in antimicrobial photodynamic inactivation? *Drug Resistance Updates,* 31, pp. 31-42.

Krishna Moorthy, A., Govindarajan Rathi, B., Shukla, S. P., Kumar, K. and Shree Bharti, V. (2021) Acute toxicity of textile dye methylene blue on growth and metabolism of selected freshwater microalgae. *Environmental Toxicology and Pharmacology,* 82, 103552.

Liu, T., Liu, Y., Fang, K., Zhang, X. and Wang, X. (2020) Transcriptome, bioaccumulation and toxicity analyses of earthworms (*Eisenia fetida*) affected by trifloxystrobin and trifloxystrobin acid. *Environmental Pollution,* 265, 115100.

Mantasha, I., Saleh, H. A. M., Qasem, K. M. A., Shahid, M., Mehtab, M. and Ahmad, M. (2020) Efficient and selective adsorption and separation of methylene blue (MB) from mixture of dyes in aqueous environment employing a Cu(II) based metal organic framework. *Inorganica Chimica Acta,* 511, 119787.

Marasini, S., Leanse, L. G. and Dai, T. (2021) Can microorganisms develop resistance against light based anti-infective agents?, *Advanced Drug Delivery Reviews,* 175, 113822.

Nitzan, Y. and Ashkenazi, H. (1999) Photoinactivation of *Deinococcus radiodurans*: an unusual Gram-positive microorganism. *Photochemistry and Photobiology,* 69, pp. 505-510.

Oliver, R.P. & Hewitt, H.G. Fungicides in crop protection. 2 ed. CABI, 2014.

513 Park, I.-S., Baek, S.-W. and Moon, K. H. (2019) The sterilization effect of methylene
514 blue, formalin, and iodine on egg and adult stage of marine medaka, *Oryzias dancena*.
515 *Development & Reproduction,* 23, pp. 199-211.

516 Peres, N. A., Timmer, L. W., Adaskaveg, J. E. and Correll, J. C. (2005) Lifestyles of
517 *Colletotrichum acutatum*. *Plant Disease*, 89, pp. 784-796.

518 Perlin, D. S., Rautemaa-Richardson, R. and Alastruey-Izquierdo, A. (2017) The global
519 problem of antifungal resistance: prevalence, mechanisms, and management. *The Lancet*
520 *Infectious Diseases*, 17, pp. e383-e392.

521 Pieranski, M., Sitkiewicz, I. and Grinholc, M. (2020) Increased photoinactivation stress
522 tolerance of *Streptococcus agalactiae* upon consecutive sublethal phototreatments. *Free*
523 *Radical Biology and Medicine*, 160, pp. 657-669.

524 Rapacka-Zdonczyk, A., Wozniak, A., Pieranski, M., Woziwodzka, A., Bielawski, K. P.
525 and Grinholc, M. (2019) Development of *Staphylococcus aureus* tolerance to
526 antimicrobial photodynamic inactivation and antimicrobial blue light upon sub-lethal
527 treatment. *Scientific Reports*, 9, 9423.

528 Reema, R. M., Saravanan, P., Kumar, M. D. and Renganathan, S. (2011) Accumulation
529 of methylene blue dye by growing *Lemna minor*. *Separation Science and Technology*,
530 46, pp. 1052-1058.

531 Rifici, L. M., Cherry, D. S., Farris, J. L. and Cairns Jr, J. (1996) Acute and subchronic
532 toxicity of methylene blue to larval fathead minnows (*Pimephales promelas*):
533 implications for aquatic toxicity testing. *Environmental Toxicology and Chemistry*, 15,
534 pp. 1304-1308.

535 Rila, J.-P. and Eisentraeger, A. (2003) Application of bioassays for risk characterisation
536 and remediation control of soils polluted with nitroaromatics and PAHs. *Water, Air, and*
537 *Soil Pollution*, 148, pp. 223-242.

538 Rocha, O. P., Cesila, C. A., Christovam, E. M., Barros, S. B. d. M., Zanoni, M. V. B. and
539 de Oliveira, D. P. (2017) Ecotoxicological risk assessment of the "Acid Black 210" dye.
540 *Toxicology,* 376, pp. 113-119.

541 Rodrigues, G. B., Dias-Baruffi, M., Holman, N., Wainwright, M. and Braga, G. U. (2013)
542 *In vitro* photodynamic inactivation of *Candida* species and mouse fibroblasts with

phenothiazinium photosensitisers and red light. *Photodiagnosis and Photodynamic Therapy*, 10, pp. 141-149.

Sabino, C. P., Wainwright, M., Ribeiro, M. S., Sellera, F. P., dos Anjos, C., Baptista, M. d. S. and Lincopan, N. (2020) Global priority multidrug-resistant pathogens do not resist photodynamic therapy. *Journal of Photochemistry and Photobiology B: Biology*, 208, 111893.

Silva-Junior, G. J., Spósito, M. B., Marin, D. R. and Amorim, L. (2014) Efficacy and timing of application of fungicides for control of citrus postbloom fruit drop, *Crop Protection*, 59, pp. 51-56.

Somsesta, N., Sricharoenchaikul, V. and Aht-Ong, D. (2020) Adsorption removal of methylene blue onto activated carbon/cellulose biocomposite films: equilibrium and kinetic studies. *Materials Chemistry and Physics*, 240, 122221.

Thai, P. K., Ky, L. X., Bihn, V. N., Nhung, P. H., Nhan, P. T., Hieu, N. Q., Dang, N. T. T., Bang Tam, N. K. and Ahn, N. T. K. (2018) Occurrence of antibiotic residues and antibiotic-resistant bacteria in effluents of pharmaceutical manufacturers and other sources around Hanoi, Vietnam. *Science of The Total Environment*, 645, pp. 393-400.

Tonani, L., Morosini, N. S., de Menezes, H. D., Bonifácio da Silva, M. E. N., Wainwright, M., Braga, G. U. L. and Von Zeska Kress, M. R. (2018) *In vitro* susceptibilities of *Neoscytalidium* spp. sequence types to antifungal agents and antimicrobial photodynamic treatment with phenothiazinium photosensitizers. Fungal Biology, 122, pp. 436-448.

United Nations. 2017. *Globally harmonized system of classification and labelling of chemicals (GHS)*. New York: United Nations.

Wainwright, M., Maisch, T., Nonell, S., Plaetzer, K., Almeida, A., Tegos, G. P. and Hamblin, M. R. (2017) Photoantimicrobials—are we afraid of the light? *The Lancet Infectious Diseases*, 17, pp. e49-e55.

Wainwright, M., Phoenix, D. A., Laycock, S. L., Wareing, D. R. A. and Wright, P. A. (1998) Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of *Staphylococcus aureus*. *FEMS Microbiology Letters*, 160, pp. 177-181.

Zhu, B., Liu, G.-L., Liu, L., Ling, F. and Wang, G.-X. (2015) Assessment of trifloxystrobin uptake kinetics, developmental toxicity and mRNA expression in rare minnow embryos. *Chemosphere*, 120, pp. 447-455.

574